THE ESTROGENICITY OF SELECTED HERBICIDES AND ADJUVANTS ENDOCRINE DISRUPTION CAPABILITIES OF SURFLAN™ AND ORYZALIN

A REPORT PREPARED FOR THE
DIVISION OF ENVIRONMENTAL ANALYSIS
CALIFORNIA DEPARTMENT OF TRANSPORTATION
INTERAGENCY AGREEMENT NOS. 43A0014 AND 43A0073

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CTSW-RT-02-039
OCTOBER 2002

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ACKNOWLEDGEMENTS

Several individuals at the California Department of Transportation have been instrumental in the development and completion of this project. This project was championed throughout by Steven Borroum, Chief of the Storm Water Program at Caltrans. Mr Borroum allowed us to pursue a study that had the potential for adverse impacts on Caltrans operations. Howard Yamaguchi shepherded the project through the early stages. Kuen Tsay assumed the role of Caltrans project manager and was extremely helpful throughout the three years of the project in securing funding in the face of significant needs elsewhere in the Storm Water Program. Dr. Dean Messer of Larry Walker and Associates served as our liaison with Caltrans and effectively communicated the importance of the study helping to maintain the source of funds. Dr. Robert Flochinni and Ms. Judy Sauer, the first Director and Assistant Director of the John Muir Institute of the Environment provided the administrative support to allow the completion of the project. Critical additional administrative support was provided by Ms Eleanor Wood of the School of Veterinary Medicine and Ms. Jennifer Nickell of the John Muir Institute.

Laboratory space and medaka exposure facilities were provided by Dr. David Hinton of Duke University, formerly of the School of Veterinary Medicine at UC Davis, Dr. Swee Teh, and Dr. Inge Werner. Considerable technical and logistic support was provided by Dr. Swee Teh, Dr. Mark Okihiro, Dr. Corrine Davis, and Dr. Christian Leutenegger. Critical review of the technical sections was provided by Dr. Swee Teh and Dr. D. Michael Fry.

EXECUTIVE SUMMARY

The research reported here was initiated at the request of the North Coast Regional Water Quality Control Board in response to citizen inquiries about the potential endocrine disruption capabilities of herbicides used by Caltrans in their roadside maintenance program. Several herbicides were considered for testing; eventually Surflan™ with the active ingredient oryzalin was selected for detailed analysis. An Endocrine Disrupting (ED) chemical can be defined as a chemical that has the ability to alter the homeostatic status of hormones or their interactions with their associated receptors (Kendall et al. 1998). There are no established guidelines to determine if a compound has endocrine disruption capabilities for a specific taxonomic group. However, there are three generally accepted criteria that lead to a conclusion of ED capabilities for the compound of interest; binding with an estrogen receptor ER, activation of an estrogen response element (ERE) on the DNA, and the resultant production of estrogen induced compounds such as vitellogenin and choriogenin. Consequently, this study concentrated on these three aspects with an additional attempt to determine if exposure to Surflan™ or oryzalin results in lesions in the gonads that might cause reproductive failure, and an attempt to establish a potential mechanistic basis for the effects.

Detection of estrogenic properties of surflan™ and oryzalin

The first experiments involved screening Surflan^{TM} and oryzalin in a stably transfected recombinant human ovarian cell line that has been validated for the identification of xenoestrogens. We then performed an analysis of receptor binding to determine if Surflan^{TM} or oryzalin could competitively displace 17-β estradiol from its receptor. Finally, *in vivo* time- and dose-response experiments with male medaka (*Oryzias latipes*) were used to ascertain whether

Surflan™ and oryzalin induce the estrogen-regulated proteins vitellogenin (Vg) and the choriogenins (Cgs).

Results from these three distinct assays support the conclusion that Surflan™ and oryzalin are ED compounds. Results from the BG1Luc4E2 reporter assay and the competitive binding assay with 17β-estradiol indicate that oryzalin and/or Surflan™ are active in these mammalian *in vitro* systems. Both Surflan™ and oryzalin were also active *in vivo*, and induced the high molecular weight choriogenins in medaka. These results are consistent with the characteristics of previously identified ED compounds such as the alkylphenols (White et al., 1994; Arukwe et al., 1997) and clearly demonstrate the ability of oryzalin and/or Surflan™ to interact with an ERE, competitively displace 17β-estradiol from the ER, and induce transcription and synthesis of the estrogen-regulated choriogenins.

Reproductive toxicity of oryzalin and Surflan™ to male and female Japanese medaka

We undertook a second study designed to determine if exposure of reproductively active, mature male and female medaka to Surflan™ or oryzalin would affect fertilization success or fecundity; viability of eggs or time-to-hatch; or cause deformities in the offspring. We also evaluated gonadal tissues of males and females histologically in order to identify and characterize any exposure-related effects.

Surflan™ and oryzalin adversely affected spermatogenesis, increasing the incidence and severity of gonadal lesions in treated animals above the levels observed in controls. The effects of

Surflan™ and oryzalin are consistent with previously reported effects of estrogens or ED compounds on spermatogenesis, in which necrotic spermatids (Miles-Richardson et al., 1999), inhibition of spermatogenesis (Billard et al., 1981; Jobling et al., 1996; Kinnberg et al., 2001, Sohoni et al., 2001), mild degeneration of testes (Lange et al., 2001), and testicular atrophy (Jobling et al., 1996; Panter et al., 1998) have been reported. Surflan™ and oryzalin could act directly at the gonad by either suppressing testosterone synthesis, altering testosterones' rate of conversion to estradiol by effects on p450arom**,** or indirectly via feedback effects at the pituitary that impact the synthesis and secretion of gonadotropins and in turn, affect steroid levels within the gonad. Exposure to Surflan™ or oryzalin affected the fertility of male and female medaka and increased the production of nonfertilized eggs (and thus decreased the production of fertilized eggs) in all treatment groups of both genders. The production of nonfertilized eggs was a highly variable event, and was typically manifested as most or all of a given clutch of eggs failing to be fertilized on the day of collection; this tended to alternate with successful fertilization for a period of days, followed by another failed fertilization. This pattern of fertilization failure was observed in all of the treated groups, and yielded fertilization data characterized by substantial variability. This variability in the response of individuals within all treatment groups made it exceptionally difficult to demonstrate statistical significance.

Exposure to both Surflan™ and oryzalin was associated with a reduced fertilization capacity of treated male medaka paired with untreated females. For males in particular, but also potentially for females, decreased fertility may be due to effects of treatment with estrogenic substances on reproductive behavior. In the brain, p450arom is concentrated in those regions linked to steroid regulation of reproduction and sexual behavior. Total daily mean egg production was not

affected by exposure to Surflan™ or oryzalin. There were statistically significant differences in the time-to-hatch of eggs from all groups of males and females treated with Surflan™ and oryzalin (Table 2-7). In all Surflan™ and oryzalin dose groups, eggs produced by untreated females paired with treated males took significantly longer to hatch than eggs from controls, and eggs produced by treated females paired with untreated males hatched significantly earlier than controls. It is difficult to speculate on the bases for the observed alterations in time-to-hatch, especially considering that egg hatching was affected differently depending on the gender of the exposed parent. Nonetheless, for exposed medaka of both genders, the effects on time-to-hatch were remarkably consistent, occurring in all dose groups. The biological significance of these effects is not clear, given that survival of progeny were not affected by exposure, and there was no effect on the incidence of deformities in juveniles grown to approximately 30 days post-hatch.

Despite the administration of greater amounts of oryzalin in the Surflan™ groups, in general, Surflan™ did not elicit more severe lesions than the lower doses of oryzalin administered alone. The one clear exception to this is seen in the prevalence and severity data for induction of intersex lesions, where Surflan™ consistently caused more severe lesions as well as a greater prevalence of these lesions than oryzalin alone. In fact, the most severe lesions often occurred in the low-dose oryzalin group, or the effects in that group were similar in magnitude to those observed from treatment with the next higher dose. Similarly, the highest concentration of Surflan™ was not associated with significant effects on non-intersex testicular lesions or on any ovarian lesion. There were marginally significant effects of high-dose Surflan™ on total egg production in treated females. These results suggest that oryzalin administered alone, or administered in Surflan™, has a distinctly non-linear dose response for the reproductive

parameters we assessed. This phenomenon has been documented for certain other ED compounds, but is not a universal characteristic of these compounds (Andersen et al, 1999; Melnick et al., 2002). It is clear from our data that we have not defined a lowest-observed-adverse- effect-level for reproductive effects of oryzalin and Surflan™.

Mechanistic indicators of toxicity of oryzalin

Estrogen is a critical regulator of reproduction in male and female vertebrates and the biological actions of estrogen, mediated by the ER, indicate that changes in ER expression have the potential to alter the transcription of estrogen-sensitive genes. Likewise, p450arom – as the primary source of endogenous estrogens – is clearly a key component in the control of estrogen-regulated gene transcription. Thus, alterations in expression of the ER and p450arom have the potential to disrupt normal reproductive function. Our reproductive study in which Surflan™ and oryzalin induced adverse effects on fertilization success, are consistent with previously documented effects of estrogen or estrogen agonists on reproduction (Billard et al. 1981; Dufau, 1988; Yasuda et al. 1985; Guillette et al. 1994; MacLatchy and Van Der Kraak 1995; Jobling et al. 1996; Toppari et al. 1996; Nimrod and Benson, 1998; Gronen et al., 1999; Gray et al. 1999a). Changes in expression of the ER and p450arom have considerable explanatory potential in understanding the adverse effects of Surflan™ and oryzalin on medaka reproduction. In this study, we used TaqMan™ PCR to quantify expression of medaka ER mRNA and p450arom mRNA. The specific objectives were to (1) characterize and quantify basal expression and chemical-induced alterations in expression of ER mRNA and p450arom mRNA in the critical reproductive tissues of brain, gonad, and liver of male and female medaka; and (2) to determine if ER mRNA and p450arom mRNA expression, measured after an exposure period of 24h, 48h,

or 21d, could be correlated with reproductive toxicity induced by 21-d exposures to Surflan™ and oryzalin (Section 2).

We demonstrated that Surflan™ and oryzalin have significant effects on the important reproductive tissues of gonad and liver. A 24h exposure to Surflan™ and oryzalin induced dramatic increases in gonadal and hepatic p450arom mRNA and ER mRNA. These effects largely reversed by 48h, and by 21d. An exception to this was seen in hepatic p450arom mRNA from 21d samples. Although the response was extremely variable and not statistically significant, our results suggest that Surflan™ and oryzalin continued to affect expression of the p450arom gene in liver for as long as 3 weeks. Few effects of Surflan™ or oryzalin were observed on whole brain ER mRNA and p450arom mRNA at any time during the experiment. We hypothesize that there may be brain-specific forms of the ER and p450arom genes that are poorly detected by our probes. Alternatively, if the respective liver ER and ovarian p450arom mRNAs are present in brain, they are minimally responsive to Surflan™ and oryzalin.

In the literature, there is documented persistence in ER induction following a single dose of estrogen (Pakdel et al., 1991; Mackay and Lazier, 1993). These results do not correspond to our results on the effects on hepatic and gonadal ER mRNA in medaka continuously exposed to Surflan™ and oryzalin. Instead of continued induction, we found an apparent compensatory down-regulation of ER mRNA began between 24 and 48h that was maintained to 21d. Two of the critical questions raised by this response are (1) what effect does the initial up-regulation of ER, or the ensuing down-regulation of ER have on gene transcription; and (2) do effects on transcription or transcription products continue once ER equilibration has taken place?

Understanding the dynamics of p450arom-generated estrogen are also integral to answering these questions; specifically, whether the initial increase in hepatic and gonadal p450arom and the presumed associated increase in estrogen have persistent effects, or whether effects are transitory, normalizing as p450arom mRNA equilibrates?

Our data demonstrate that Surflan™ and oryzalin elicit a spectrum of biological effects in medaka, and do so after exposure to the same concentration of oryzalin that impaired reproduction and caused gonadal lesions following a 21d exposure. Although many questions remain concerning the mechanistic basis of Surflan™ and oryzalin's toxicity, our results suggest that perturbations in estrogen synthesis and ER dynamics may be circumstantially linked to the reproductive toxicity caused by these compounds.

Relevance of results

One of the main unresolved questions raised by our results is if the effects found in this investigation are occurring at concentrations that are environmentally relevant, i.e., are concentrations found in natural systems comparable to the dose levels used in this study. Because herbicides are assumed to be essentially nontoxic to mammals and birds, and only slightly toxic to fish, few studies have attempted to measure the concentrations of Surflan™ or oryzalin in the field. The only study with reliable data is the study currently being conducted by T. Young on the runoff of oryzalin from sites near Tolay Creek and the Eel River. Over two years, concentrations of oryzalin in storm water runoff from the Tolay Creek site averaged 2.2 µg/L and 0.7 µg/L (EMC) while concentrations at the Eel River site averaged 12.6 µg/L and 17.9 µg/L (EMC). Single storm EMC concentrations were found to be as high as 43 µg/L. Effects

were seen in this study at concentrations as low as 250 µg/L, well above the levels measured by Young et al. (unpubl) in their field study. Our studies were conducted after a general dose-range finding process, and it was unknown at the time the studies were initiated what the environmentally relevant concentrations would be. Consequently, no attempt was made to test for effects at concentrations as low as those seen in the field. However, because we have not defined a lowest-observed-adverse- effect-level for reproductive effects of oryzalin and Surflan™, and because there appears to be a nonlinear dose-response relationship, and because effects are seen within 24 hours of exposure, it is critical that our studies be repeated at the low concentrations detected by Young et al. in their study.

INTRODUCTION

Before chemicals are used as pesticides in the environment, they are rigorously screened for potential toxicity to a wide variety of plants and animals. This screening typically covers effects on direct survival and reproduction, and a series of effects such as mutagenicity and teratogenicity. The testing procedure required for registration was developed prior to the discovery of the potential for chemicals to cause endocrine disruption (ED). Within the last 10 years, it has been found that once many chemicals enter the body, they can mimic the action of hormones causing severe disruption of reproduction and development. These effects are not noticeable during standard toxicity tests and may not occur for a considerable period of time after exposure. In fact, many of the effects are realized in offspring of animals exposed to ED chemicals. The objective of this project is to determine if herbicides used by Caltrans as part of normal maintenance activities have any endocrine disruption capabilities.

The research reported here was initiated at the request of the North Coast Regional Water Quality Control Board in response to citizen inquiries about the potential endocrine disruption capabilities of herbicides used by Caltrans in their roadside maintenance program. Several herbicides were considered for testing; eventually Surflan™ with the active ingredient oryzalin was selected for detailed analysis. The investigation consisted of three steps that included 1) an initial screening to determine if both compounds possessed endocrine disruption capabilities, 2) a reproductive toxicity test with accompanying histopathologic examination of gonads to determine if the compounds disrupted reproductive function, and 3) an analysis to determine the potential mechanism for the endocrine disruption activity.

A major focus of this project was development of methods suitable for the testing of compounds for ED activity. We focused on developing test procedures using Japanese medaka, a standard laboratory organism. Advantages of the medaka include rapid generation time allowing reproductive and developmental studies in a short period of time, ease of husbandry, and a well-established body of literature on all aspects of their biology. Medaka are currently used in numerous studies of ED in fish.

ENDOCRINE DISRUPTING CHEMICALS

An ED chemical can be defined as a chemical that has the ability to alter the homeostatic status of hormones or their interactions with their associated receptors (Kendall et al. 1998). Although often assumed to affect only reproductive function, these compounds can affect endocrine hormones and signal transduction pathways that are critical to growth and survival (Kendall et al. 1998). ED chemicals fall into several categories including estrogen agonists (e.g., some PCB congeners and DDT), estrogen antagonists (e.g., dioxin) and androgen antagonists (e.g., vinclozalin). Estrogenic chemicals vary considerably but usually include a sterically unrestricted phenolic group and a hydrophobic substituent of greater than 4 carbons bonded opposite the phenolic group (Giesy and Snyder 1998). These chemicals can cause effects in almost every vertebrate group including fish.

There are no established guidelines to determine if a compound has endocrine disruption capabilities for a specific taxonomic group. However, there are three generally accepted criteria that lead to a conclusion of ED capabilities for the compound of interest; binding with an

estrogen receptor, activation of an estrogen response element on the DNA, and the resultant production of estrogen induced compounds such as vitellogenin and choriogenin. Consequently, this study concentrated on these three aspects with an additional attempt to determine if exposure to Surflan™ or oryzalin results in lesions in the gonads that might cause reproductive failure, and an attempt to establish a potential mechanistic basis for the effects.

TOXICITY OF OYRZALIN (TAKEN FROM PESTICIDE INFORMATION PROFILES LOCATED ON THE EXTENSION TOXICOLOGY NETWORK [1996])

Surflan™ is a proprietary herbicide emulsion formulated and marketed by Dow Elanco. It contains oryzalin (3,5-dinitro-N4, N4-dipropylsulfanilamide) as the active ingredient (40.4%). Oryzalin is a selective, pre-emergence, surface-applied herbicide used for the control of annual grasses and broadleaf weeds. Oryzalins' herbicidal action has been attributed to its ability to block cell division in plant meristematic tissue (Kidd and James, 1991). In California, oryzalin is used on a wide variety of crops, as well as in urban areas and on roadsides for weed control. From 1991to 2000, usage of oryzalin ranged from 814,397 lbs on 403,701 acres in 1998 to a low of 456,521 lbs on 219,040 acres in 2000; the application rate has been relatively constant at about 1.6 to 1.7 lbs per acre (PAN, 2002).

Oryzalin (3,5-dinitro-N4, N4-dipropylsulfanilamide, see Appendix I for physical-chemical profile) is a selective pre-emergence surface-applied herbicide used for control of annual grasses and broadleaf weeds. It inhibits growth by blocking cell division in meristematic tissue. Oryzalin is practically nontoxic to mammals and birds by ingestion, with reported oral LD50 values of greater than 5000 mg/kg in rats and mice (Kidd and James 1991, Weed Science Society

of America 1994), and greater than 1000 mg/kg in cats, dogs, and chickens (Kidd and James 1991, USEPA 1987). The dermal LD50 for technical oryzalin in rabbits is greater than 2000 mg/kg, indicating slight to practically no toxicity by this route (Weed Science Society of America 1994). It is reported to cause slight skin and eye irritation in the rabbit, and no skin sensitization in the guinea pig (Weed Science Society of America 1994). It is also slightly toxic when inhaled, with a 4-hour inhalation LC50 of greater than 3 mg/L in rats (Weed Science Society of America 1994). The formulated products (e.g., Surflan A.S.) may show moderate toxicity by either the oral or inhalation routes, and may show skin and eye irritation and skin sensitization properties (Weed Science Society of America 1994). In dogs and cats, large oral doses cause nausea and vomiting (U.S. National Library of Medicine 1995).

Rats fed a dietary level of about 2.5 mg/kg/day for 2 years exhibited blood changes, increased liver and kidney weights, inhibition of growth, and decreased survival (U.S. National Library of Medicine 1995). Repeated ingestion of large doses led to adverse changes in blood cell formation in dogs (U.S. National Library of Medicine 1995). Mice given dietary doses of about 200 mg/kg/day for 1 year exhibited decreased uterine and ovarian weights. Those exposed to doses of 75 mg/kg/day showed no observable effects (USEPA 1990).

There were no adverse effects on reproduction in a three-generation study of rats fed dietary concentrations of 12.5, 37.5, or 112.5 mg/kg/day, the highest dose tested. Fetotoxic effects appeared at 12.5 mg/kg/day (Weed Science Society of America 1994, USEPA 1990). It does not appear that oryzalin causes reproductive effects. There were no birth defects in the offspring of pregnant rats fed dietary concentrations as high as 112 mg/kg/day for three generations, or in the

offspring of pregnant rabbits given doses of 125 mg/kg/day (U.S. National Library of Medicine 1995, USEPA 1990). It appears that oryzalin is unlikely to cause teratogenic effects. Oryzalin was not mutagenic in several tests, including tests on live rats and mice and on bacterial cell cultures (USEPA 1990). It does not appear that oryzalin is mutagenic. When oryzalin was fed to rats in doses as high as 135 mg/kg/day for 2 years, there was an increase in the incidence of thyroid, mammary, and skin tumors (USEPA 1990). Thyroid tumors and benign skin and mammary tumors occurred in rats fed a dietary level of 45 mg/kg/day for 2 years [107]. However, there were no tumors in mice fed doses as high as 548 mg/kg/day for 2 years (USEPA 1990). Because of these conflicting results, it is not possible to assess the carcinogenicity of oryzalin. Oryzalin has shown systemic effects on the thyroid, liver, and kidneys, as well as blood chemistry, in animal tests.

The reported oral LD50 values in bobwhite quail and mallard ducks are greater than 500 mg/kg, and in chickens is 1000 mg/kg (Kidd and James 1991, Weed Science Society of America 1994). The 5-day dietary LC50 values in quail and mallard ducks are greater than 5000 ppm (U.S. National Library of Medicine 1995). Oryzalin is highly toxic to fish, with reported 96-hour LC50 values of 2.88 mg/L in bluegill sunfish, 3.26 mg/L in rainbow trout, and greater than 1.4 mg/L in goldfish fingerlings (U.S. National Library of Medicine 1995).

No breakdown of oryzalin by hydrolysis was observed at pH 5, 7, and 9 (U.S. National Library of Medicine 1995). Based on its behavior in soil, breakdown by microbial processes is probably slow in the aquatic environment due to low levels of oxygen and low microbial activity. Photodegradation may be significant in the upper portions of the water column.

SECTION I

DETECTION OF ESTROGENIC PROPERTIES OF SURFLAN™ AND ORYZALIN

A spectrum of adverse reproductive and behavioral effects have been linked with exposure to chemicals that act to disrupt one of numerous components of the endocrine system. Referred to generally as endocrine disrupting chemicals or endocrine disruptors (ED's), these chemicals share the ability to alter endocrine homoeostasis in the exposed animal (for reviews see Nimrod and Benson, 1996; Andersen et al., 1999; Guillette and Gunderson, 2001). ED's have been very broadly defined as any exogenous agent that interferes with the production, release, transport, metabolism, binding, biologic action, or elimination of endogenous hormones (Zacharewski et al., 1998). The focus of both the regulatory and scientific communities has been on those ED's that mimic the effects of endogenous estrogens. Those substances are referred to here as xenoestrogens or as having estrogenic activity. Xenoestrogens identified to date include various phytoestrogens (e.g., β-sistosterol, MacLatchy and Van Der Kraak, 1995), pharmaceutical estrogens (Majdic et al., 1996; Scholz and Gutzeit, 2000; Bjerselius et al., 2001), the plasticizer bisphenol A (Ramos et al., 2001), surfactant degradation products (Jobling et al., 1996), and DDT and certain of its metabolites (Fry et al., 1981; Stewart et al., 2000).

Specific biochemical mechanisms' of action have generally not been fully characterized for xenoestrogens, but effects include alterations in expression of critical steroidogenic enzymes (Majdic et al., 1996; Sholtz and Gutzeit, 2000) perturbation of the hypothalamo-pituitary-gonadal axis (MacLatchy and Van Der Kraak, 1995; Blake and Boockfor, 1997), alterations in

plasma sex steroids (Folmar et al., 1996; 2000), and effects on reproductive behavior (Bjerselius et al., 2001).

Xenoestrogens are structurally diverse and often have little apparent similarity to the principal endogenous estrogen of vertebrates, 17-β estradiol. Xenoestrogens commonly bind to the estrogen receptor (ER), a protein in the nuclear receptor superfamily. Unique among nuclear receptors, the ER has the ability to bind a number of structurally dissimilar chemicals. This capacity to bind a heterogenous group of ligands has been partially attributed to the size of the ligand-binding pocket of the receptor, which is substantially larger in volume than 17-β estradiol (Brzozowski et al., 1997). In this report, we add a new chemical, in a new structural class, to the list of chemicals with estrogenic activity.

We conducted a series of *in vitro* and *in vivo* experiments to evaluate whether Surflan™ or oryzalin have estrogenic activity. The first experiments involved screening Surflan™ and oryzalin in a stably transfected recombinant human ovarian cell line that has been validated for the identification of xenoestrogens. Rogers and Denison (2000) constructed a reporter plasmid vector containing the firefly luciferase gene under hormone-inducible control of estrogen response elements (ERE). That vector, stably transfected into the estrogen receptor-containing human ovarian cell line BG-1 yielded the recombinant cell line, BG1Luc4E2. This cell line responds to 17-β estradiol, as well as to a number of structurally unrelated xenoestrogens, by induction of fluorescent luciferase. The second xperiment was a receptor-binding study investigating if Surflan™ or oryzalin could competitively displace 17-β estradiol from its receptor. Finally, *in vivo* time- and dose-response experiments with male medaka (*Oryzias*

latipes) were used to ascertain whether Surflan™ and oryzalin induce the estrogen-regulated proteins vitellogenin (Vg) and the choriogenins (Cgs).

Materials and Methods

Detection of Estrogenic Activity of Surflan™ and Oryzalin using a Stably Transfected Human Ovarian Cell Line

BG1Luc4E2 cells were plated in 24-well plates and cultured in estrogen-stripped media without phenol red (Sigma) supplemented with 5% dextran-coated charcoal treated fetal bovine serum (Hyclone) for seven days, with the media being changed daily. At approximately 90% confluence, cells were treated with the compound of interest [(Surflan™ or oryzalin (DowElanco), 17-β estradiol (Sigma), or dimethylsulfoxide (Sigma)] in media for 24 hours. The media was removed, the plates were rinsed with phosphate-buffered saline, and cells lysed with 100µl of lysis buffer (Promega). Lysed samples were collected and the cell debris was pelleted by centrifugation. Luciferase activity in 25µl of lysate + 50µl of luciferase reagent (Promega) was measured in a Dynatech ML3000 microplate luminometer. Luciferase activity was normalized to the protein concentration of the cell lysate using the fluorescamine assay (Kennedy et al., 1995) and bovine serum albumin as the standard. Briefly, 100µl of fluorescamine (500 µg/l in acetonitrile) was added to each microplate well, the plate was agitated for 20 minutes in the dark, and fluorescence was measured in a Fluostar plate reader with excitation and emission wavelengths of 390 and 460 nm, respectively. All assays were conducted with at least three replicate measurements for each chemical concentration.

Estrogen Receptor Binding

Calf uterine cytosol (12.5 mg/ml protein) was incubated with 5nM 17-β estradiol ([6,7-^{3}H(N)]-estradiol, 47.2 Ci/mmol, New England Nuclear Life Sciences, Inc.) (total binding) or with 5nM

[^{3}H] estradiol and 200nM diethylstilbestrol (Sigma)(non-specific binding), oryzalin (Dow Elanco, 100µM) or Surflan™ (Dow Elanco, 1:10,000 dilution). Cytosol samples were incubated for 2h at 4°C, then added to pellets of dextran-coated charcoal (Sigma), vortexed, and incubated at 4°C for 15 minutes. After centrifugation at 3500 rpm for 10 minutes, 300 µl of supernatant (0.96 mg total protein) was added to 5 ml of liquid scintillation fluor (Sigma), and disintegrations per minute were determined by liquid scintillation counting. Specific binding was calculated as the difference between total binding and non-specific binding, adjusted for sample protein content and the specific activity of the radiolabeled estradiol.

Induction of Choriogenin and Vitellogenin

Male medaka were exposed to oryzalin (DowElanco) or Surflan™ (Dow Elanco) in two separate experiments. In the first study, seven-month-old male medaka were isolated from females and placed in static, aerated tanks for 14d prior to exposure to reduce the likelihood of protein induction from solubilized estrogens released by females. At the end of the isolation period, medaka were placed in 1-L aerated beakers, containing U.S. EPA reconstituted water (Horning and Weber, 1985) at pH 7.2. Medaka were bath exposed for 3 d in solutions (nominal concentrations) of 17-β estradiol (15 ng/ml, Sigma Chemical Co.), Surflan™ (8.5, 5.5, or 3.8 µl/l) or oryzalin (3.3, 2.2, or 1.5 mg/l). Exposure concentrations were selected from an initial range-finding experiment in which medaka were exposed to nominal concentrations of Surflan™ (34, 17, 8.5, 5.5, 3.8, 2.5, 1.3, or 0.67 µl/l) or oryzalin (3.3, 2.2, 1.5, 1.0, 0.5, or 0.25 mg/L) for three days. The apparent three-day Maximum Tolerable Dose (MTD) was selected as the concentration for the high-dose group for the protein induction experiments, with the next two lower concentrations selected as the mid- and low-dose groups, respectively.

Each treatment was comprised of three replicates, using five medaka per replicate. Animals were maintained at approximately 25°C under a photoperiod of 16h light, 8h dark. Light was provided at 400-450 lux by Vita-Lite (Coralife Chromatic™) bulbs. Medaka were fed live brine shrimp at approximately 3.5% of their bodyweight per day. Exposure solutions were made fresh daily, with the solution in each beaker completely replaced each day.

In the second experiment, six-month-old male medaka were isolated prior to exposure as described above. At the end of the isolation period, three replicates of medaka (n = 4)were placed in 1-L solutions of Surflan™ (2.5, 1.3, or 0.67 µl/l), oryzalin (1.0, 0.5, or 0.25 mg/l), 17-β estradiol (15 ng/ml), or reconstituted water. Exposure concentrations were selected from data of range finding studies as previously described, except that range-finding studies were continued for 16 days. The apparent MTD for a 16-d exposure period was selected as the concentration for the high-dose group, with the next two lower concentrations selected as previously noted. Exposures were maintained for 16 d under temperature, light, and feeding conditions given above. The contents of each beaker were completely replaced on a daily basis with freshly prepared solutions.

Medaka were euthanized with tricaine methane sulfonate (MS222), livers dissected, frozen in liquid nitrogen, and then stored at –80°C for later use. To prepare tissues for protein separation and analysis, livers were pooled from each treatment replicate (n= 4 or 5), homogenized on ice in a buffer containing 50 mM Tris pH 7.6, 12 mM monothioglycerol, 1.0 mM ethylenediaminetetraacetic acid, 1.0 mM dithiothreitol, and 20% glycerol (Sigma, St. Louis,

MO). Immediately before use, a single protease inhibitor tablet (Roche Molecular Biochemicals) was added to 10 ml of the homogenate.

The homogenate was centrifuged at 10,000 rpm for 45 minutes; the supernatant was transferred to a new sample tube and centrifugation was repeated for two, 30-min periods. Protein concentrations of each pooled sample were measured on a plate reader (Molecular Devices Corporation) using the Lowry method with slight modifications. Reagents used in protein concentration measurements were purchased from BioRad Laboratories. Liver proteins were separated by electrophoresis on a precast 10% Tris-HCl gel (BioRad Laboratories) with prestained SDS-PAGE high-range standards (BioRad Laboratories), then transferred by electrobloting to an Immobilon-P nitrocellulose membrane (Millipore) in a transfer buffer of 0.01% sodium dodecyl sulfate, 10% methanol, 1.92M glycine, and 0.25M Tris base. Membranes were then placed in a solution containing 5% skim milk powder, 0.5 ml Tween 20, 15mM Tris, and 0.5M NaCl, and allowed to equilibrate for 30 min. Unless otherwise noted, all chemicals were obtained from Sigma.

Membranes were incubated for 1.5 h with either a monoclonal antibody to vitellogenin (1:1000 dilution, mouse anti-striped bass vitellogenin, Cayman Chemical Company) or with a polyclonal antibody to choriogenins (1:2000 dilution, rabbit anti-medaka choriogenin, (provided by K.Murata, University of California, Davis). After three, 30-min washes in transfer buffer (described above) supplemented with 0.5 ml Tween-20, membranes were reacted with either a goat anti-mouse IgG Alkaline Phosphatase conjugate secondary antibody (1:30,000 dilution, Sigma Chemical Co.) for vitellogenin detection, or a goat anti-rabbit IgG Alkaline Phosphatase

conjugate secondary antibody (1:7500 dilution, BioRad Laboratories) for choriogenin detection. Proteins were visualized by reaction with CDP-Star^{R} substrate (Applied Biosystems).

Statistical Calculations

Statistical analysis of the data was performed using Microsoft EXCEL® and Number Cruncher Statistical System® (NCSS). Student's t-Test was used to determine the significance of the differences between treatments and controls.

Results

Detection of Estrogenic Activity of Surflan^{TM} and Oryzalin in BG1Luc4E2 cells

Surflan^{TM} incubated with BG1Luc4E2 cells for 24h induced significant luciferase activity when tested at dilutions of 1:10,000 ($p = 0.02$) or 1:100,000 ($p = 0.002$). A 1:1000 dilution of Surflan^{TM} was lethal to cells (data not shown), and a 1:1,000,000 dilution of Surflan^{TM} gave no indication of estrogenic activity (Figure 1-1). Incubation of oryzalin at concentrations of 5 µg/ml and 0.5 µg/ml induced significantly higher levels of luciferase in BG1Luc4E2 cells than DMSO controls ($p = 0.02$). Lower concentrations of oryzalin were not effective (Figure 1-2).

Oryzalin was also incubated with BG1Luc4E2 cells alone, or in combination with 1nM 17-β estradiol (Figure 1-3). Again, oryzalin tested at 5 µg/ml and 0.5 µg/ml induced significant luciferase activity ($p < 0.03$, $p = 0.04$, respectively). All concentrations of oryzalin in combination with 1 nM estradiol also induced luciferase activity ($p < 0.01$). However, this induction was less than that observed with estradiol alone, and indicates that oryzalin partially inhibits the biological activity of estradiol in BG1Luc4E2 cells.

Estrogen Receptor Binding

Oryzalin (100 μM) competitively displaced 17-β estradiol from the estrogen receptor, inhibiting 17-β estradiol's binding by ~35% ($p = 0.01$). Surflan™ (1/10,000 dilution) did not displace 17-β estradiol (Figure 1-4).

Induction of Choriogenin and Vitellogenin

17-β estradiol and high-dose oryzalin (3.3 μg/ml) and Surflan™ (8.5 μl/l) induced production of choriogenins (Cgs) in male medaka. No induction of Cgs was observed at lower concentrations, (Figure 1-5). Vitellogenin was detected in male medaka exposed to 17-β estradiol for three days (Figure 1-6) and 16 days (data not shown), but was not induced by oryzalin or Surflan™ used in these experiments regardless of exposure duration.

Discussion

We have used a reporter gene expression assay and a competitive ligand binding assay to demonstrate binding of Surflan™ and oryzalin to an ERE, and competitive displacement of 17-β estradiol from it's receptor by oryzalin. Both Surflan™ and oryzalin induced high molecular weight Cgs in male medaka, but neither chemical affected expression of Vg.

Intracellular receptor proteins present in the liver, reproductive tract, brain, and other estrogen-responsive tissues mediate the biological effects of estrogen. In many vertebrates, more than one form of the ER exists (Paech et al., 1997; Kuiper et al., 1997; Hawkins et al., 2000; Klinge, 2001). Regardless of the specific isoform, all ER's identified to date are transcription factors

activated by binding of estrogen or an estrogen agonist to the receptor. Once ligand binding occurs, the ligand-ER complex interacts as a homodimer with an ERE and modulates gene expression (Pakdel et al., 1991; Klinge, 2001).

Estrogen receptors are characterized by their ability to bind 17-β estradiol and other estrogens (e.g., diethylstilbestrol) with high affinity (Evans, 1988). Competitive displacement of 17-β estradiol from the ER by a non-estrogen yields evidence that the compound has both an affinity for the ER and has the ability to chemically displace estradiol from its receptor (Mueller and Kim, 1978). The biological significance of this activity is not explicitly understood, but includes the possibility that the non-estrogen•ER complex can interact with ERE(s) and activate or suppress the transcription of estrogen-regulated genes. Indeed, the combination of evidence that we present here suggests that oryzalin does displace 17-β estradiol from the ER; that the oryzalin•ER complex can bind to an ERE; and that the oryzalin•ER complex can initiate the transcription of the estrogen regulated genes, the Cg's (see below).

The BG1Luc4E$_2$ bioassay is a highly sensitive reporter gene assay that has been validated with known xenoestrogens and has provided preliminary identification of two novel estrogenic polychlorinated biphenyls (Rogers and Denison, 2000). Equally important, the BG1Luc4E$_2$ cells also respond accurately (i.e., negatively) to non-estrogenic chemicals. The positive results obtained with the BG1Luc4E$_2$ assay demonstrate the ability of Surflan^{TM} and oryzalin to activate the ERE-mediated signaling system specific to these recombinant cells. Although these data do not discriminate between direct and indirect activation of ERE's, the complimentary results of

the competitive binding assay strongly suggest that oryzalin directly binds to the ER, and that the ligand-receptor complex does interact directly with the ERE.

A relatively high concentration of oryzalin (100 μM) was selected for the ligand-binding assay to maximize our ability to observe competitive displacement of 17-β estradiol from the ER. This assay utilized dextran-coated charcoal to separate bound and free ligand as described in EORTC (1973) and Thorpe (1987). A 1:10,000 dilution of Surflan™ (nominal concentration of 140μM oryzalin) gave near-maximal luciferase activity in BG1Luc4E_2 cells, yet was inactive in the competitive binding assay (Figure 1-4). As an emulsion, the actual solubility of Surflan™ under the conditions of the competitive binding assay is unknown, and may be markedly different than the nominal (target) concentration. Furthermore, even if approximately similar concentrations of oryzalin in Surflan™ and oryzalin used alone were achieved, the physical characteristics of the emulsion may alter (decrease) the rate of uptake of Surflan™, reducing our ability to detect competitive displacement of 17-β estradiol during the short reaction time (approximately 2h) of the assay.

Xenoestrogens typically have a much lower affinity for the ER than does 17-β estradiol (Andersen et al., 1999). This relationship appears to hold for oryzalin also, as notwithstanding the high concentration used to evaluate binding to the ER, only modest competitive displacement of 17-β estradiol was observed. The apparent relatively low affinity of oryzalin for the ER is consistent with the substantially lower activation of the reporter gene luciferase by Surflan™ or oryzalin relative to 17-β estradiol observed in the BG1Luc4E_2 assay (Figures 1-1, 1-2), and is also consistent with the data of Figure 3. In the latter figure, oryzalin is shown to partially inhibit the

expression of ERE-dependent luciferase in BG1Luc4E$_2$ cells co-incubated with 17-β estradiol. One interpretation of these data is that when oryzalin binds to endogenous ERs present in the BG1Luc4E$_2$ cells, it does so with less affinity for those ERs than 17-β estradiol and consequently, is less efficient at inducing luciferase expression. However, because there are a finite number of ERs, those ERs occupied by oryzalin are not available for binding by 17-β estradiol, resulting in a net lower activation of luciferase when the two compounds are assayed together.

One of the most biologically significant responses to estrogens is the control of protein transcription and translation (Tata and Smith, 1979). In male teleosts, perhaps the most thoroughly characterized response to xenoestrogens is the induction of Vg (Le Guellec et al., 1988; Pelissero et al., 1993; Heppel et al., 1995; Sumpter and Jobling, 1995; Ren et al., 1996; Thompson et al., 2000; Cheek et al., 2001), the egg yolk precursor protein normally synthesized only by the liver of reproductively active females in response to endogenous estradiol. Vg is secreted by the liver into the blood, transported to the ovary, and modified into the yolk proteins lipovitellin and phosvitin (Wallace and Sellman, 1981). These proteins are stored in the yolk and serve as food reserves for the developing embryo (Chester-Jones et al., 1987).

The Cgs are estrogen-regulated egg envelope proteins also synthesized in the liver of teleosts, secreted into the plasma and taken up by the ovary (Oppen-Berntsen et al., 1992; Hyllner et al., 1991; Murata et al., 1995; 1997a, b). In fish, egg envelope formation occurs during oogenesis. The completed envelope (chorion) of a full-grown oocyte consists of one or two thin outer layers and a single thick inner layer. The inner layer of the medaka egg envelope is composed of subunits with molecular weights ranging from 74-76,000 (the high molecular weight Cg's) and a

unit of 49,000 (low molecular weight Cg) (Hamazaki et al., 1987 a, b; Murata et al., 1993, 1995, 1997a,b). The precursor proteins of the inner layer of the egg envelope of medaka have been characterized and named choriogenin H and L for the high- and low-molecular weight precursor proteins, respectively (Hamazaki et al., 1987 a, b; Murata et al., 1991,1993, 1995, 1997a,b). The polyclonal medaka Cg antibody used in our experiments was generously provided by K.Murata, currently at the University of California, Davis. That antibody reacts specifically with the high molecular weight Cg's, as well as with additional unidentified proteins in ascites fluid or liver of 17-β estradiol-treated medaka (Murata et al., 1991; 1993). In our western blots from oryzalin- and 17-β estradiol-treated medaka, the antibody also reacted with proteins other than the Cg's it was developed against. The identity of these proteins is not known.

Fish are often the model of choice for the initial characterization of a chemicals' ability to modulate expression of estrogen-regulated genes. Vg and Cg genes are present in male teleosts, and their expression can be induced by exposure to estrogen or estrogenic EDs (Le Guellec et al., 1988; Murata et al., 1991; 1993; Pelissero et al., 1993; Heppel et al., 1995; Sumpter and Jobling, 1995; Ren et al., 1996; Celius and Walther, 1998; Gronen et al., 1999; Thompson et al., 2000; Cheek et al., 2001). Very little, if any, Vg or Cg can be detected in unexposed adult male teleosts, probably because of low levels of circulating estrogens. However, occasional detection of Vg in unexposed males indicates that there may be considerable variability in the levels of circulating 17-β estradiol in males and/or that estrogen released by females into aquaria water may be sufficient to induce low levels of these proteins in males (Tyler et al., 1996; Panter et al., 1998; Tyler et al., 1999). To minimize the influence of female-derived estrogens, male medaka in our Vg and Cg experiments were isolated for 14d prior to chemical exposure. Livers from

control males in these experiments showed no evidence of either protein as detectable by western blotting.

At the present time, we do not understand the inability of Surflan™ and oryzalin to induce Vg in adult male medaka. The striped bass anti-Vg monoclonal antibody used has been validated for the detection of Vg in medaka (Gronen et al., 1999), and its specificity and activity in our assays were confirmed by the induction of medaka Vg by 17-β estradiol. Constant aqueous exposure, such as we describe here, has been shown to be more effective than other exposure routes for Vg gene activation and synthesis (Bowman et al., 2000). Published reports indicate that the maximum induction of Vg and Cg proteins occur between seven and 12 days (Shapiro et al., 1976; Le Guellec et al., 1988; Lech et al., 1996; Sherry et al., 1999; Korte et. al., 2000), and that the time course for maximum induction of Vg mRNA ranges from 16h to 15d (Limm et al., 1991; Pakdel et al., 1991) – time periods largely encompassed by our three- and 16-day exposures. Furthermore, although Thompson et al. (2000) reported that the EC_{50} concentrations of 17-β estradiol required to induce Vg varied nearly 8-fold between medaka, sunshine bass (*Morone saxtalis* x *Morone chrysosps*) and channel catfish (*Ictalurus punctatus*); medaka was the most sensitive of these three species.

Although there is a substantial body of scientific data that documents the utility of Vg as a biomarker of exposure to structurally-diverse estrogenic substances (Le Guellec et al., 1988; Pakdel et al., 1991; Pelissero et al., 1993; Heppel et al., 1995; Sumpter and Jobling, 1995; Ren et al., 1996; Gronen et al., 1999; Thompson et al., 2000), recent reports indicate that it may be a less sensitive marker of estrogenicity than Cg. For example, male Atlantic salmon exposed to

different doses of the ED nonylphenol (NP) responded by production of a Cg at all doses tested (1 to 125 mg/kg, i.p.), yet Vg synthesis was stimulated only in the highest dose group (125 mg/kg) (Arukweke et al., 1997). Similarly, o,p'-DDT induced Cgs in Atlantic salmon but not Vg (Celius and Walther, 1998a). In a separate study, although estradiol induced both Vg and Cgs in Atlantic salmon (Celius and Walther, 1998b, Cg induction occurred sooner (one week vs. two weeks post-injection) and in response to lower doses of estradiol than Vg. Similarly, Cg mRNA induction was more responsive to low-levels of 17-β estradiol than Vg in rainbow trout (Celius et al., 2000).

The universal utility of Vg as a biomarker of estrogenicity has also been brought into question by the data of Cheek et al. (2001), who concluded that induction of Vg was the least sensitive of the physiologic responses measured in medaka exposed to the estrogenic pesticide o,p'-DDT. Specifically, o,p'-DDT had no effect on Vg expression after a two-week exposure, yet all doses induced Vg expression when exposures were continued for eight weeks. Hemmer et al. (2001) found that sheepshead minnows produced neither Vg mRNA nor detectable levels of Vg in serum of males exposed to the estrogenic pesticide endosulfan, although NP or methoxychlor induced Vg mRNA and serum Vg in a dose-dependent manner. Thus, the sensitivity of Vg induction as a biomarker of estrogenicity appears to be species-, time- and chemical-dependent. The underlying basis for this variability is not known, but may include species-specific differences in the number of EREs on the Vg gene, the number of ERs present in the liver of different species, or chemical-specific differences in ER binding affinity. Both Hyllner et al. (1994) and Celius and Walther (1998b) demonstrated that during fish oogenesis, the formation of the egg envelope proteins is initiated before vitellogenesis – a time when levels of endogenous

levels of estrogen are lower. The hypothesis that Cgs are expressed in response to lower levels of estrogen than Vg (and by extension, lower levels of xenoestrogens) may partially explain the differing response to discrete estrogenic chemicals, although confirmation of this will require a determination of the chemicals' relative estrogenic potencies.

As a marker of estrogenicity, Cg induction may also have greater biological significance than the induction of Vg. Experimentally induced expression of Vg has been causally linked to renal damage (Folmar et al., 2001), presumably from an inability to clear the massive amount (e.g., million-fold increase) of protein produced (Jobling et al., 1996). Adverse effects of Vg from environmentally relevant concentrations of xenoestrogens have not been demonstrated. Although the consequences of Cg induction remain unknown, it is plausible that perturbation of Cg production in females may alter the integrity of the egg envelope, making it more susceptible to mechanical damage or polyspermy, and ultimately, susceptible to problems with hatching and survival (Aruweke et al., 1997; Arukweke et al., 2000).

Conclusion

In this section, data are presented that identified Surflan™ and it's active ingredient oryzalin as xenoestrogens. These results are the first to identify a dinitrosulfanilamide as having estrogenic activity.

Although no specific criteria have been established to irrefutably identify a chemical as a xenoestrogen [see Zacharewski (1997) for a discussion of possible scientific criteria], we have presented results from three distinct assays in support of that conclusion. Results from the

BG1Luc4E2 reporter assay and the competitive binding assay with 17-β estradiol indicate that oryzalin and/or Surflan™ are active in these mammalian *in vitro* systems. Both Surflan™ and oryzalin were also active *in vivo*, and induced the high molecular weight Cgs in medaka. These results are consistent with the characteristics of previously identified xenoestrogens such as the alkylphenols (White et al., 1994; Arukwe et al., 1997) and clearly demonstrate the ability of oryzalin and/or Surflan™ to interact with an ERE, competitively displace 17-β estradiol from the ER, and induce transcription and synthesis of the estrogen-regulated Cgs.

As xenoestrogens, Surflan™ and oryzalin have the potential to adversely affect those biological processes controlled by endogenous estrogen. In teleosts, estrogen regulates many processes critical to reproduction, including vitellogenesis and choriogenin synthesis (Chester-Jones et al., 1987; Murata et al., 1994, 1997a,b), reproductive behavior (Gray et al., 1999, Bjerselius et al., 2001), and potentially, spermatogenesis (Bouma and Nagler, 2001). In the following sections, experimental results are presented from studies with medaka that examined the reproductive effects of exposure to Surflan™ and oryzalin (Section 2), and evaluated the ability of Surflan™ and oryzalin to perturb estrogen homeostasis (Section 3).

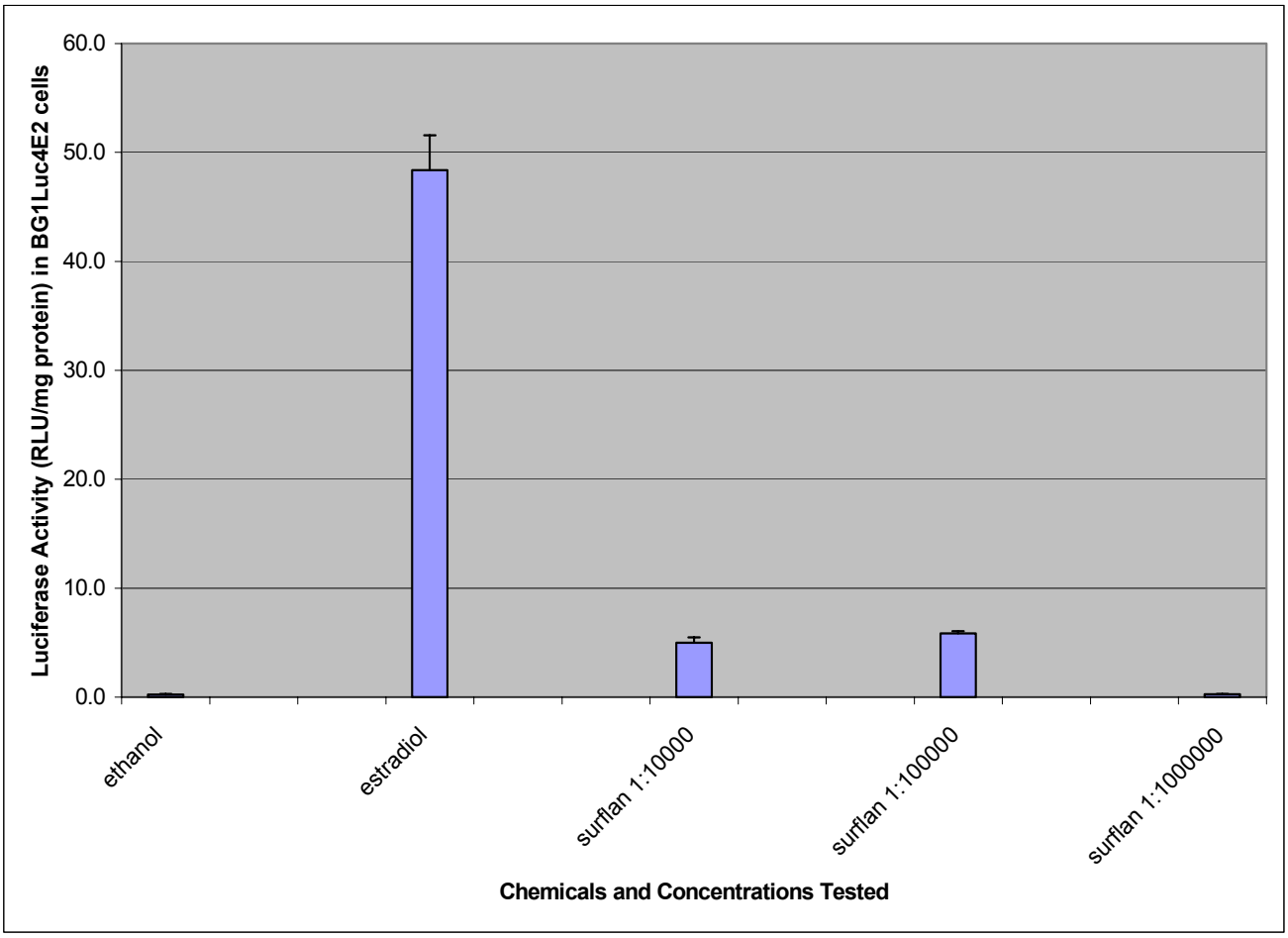

Figure 1-1. Estrogenic Activity of Surflan™ in BG1Luc4E2 Cells. Values represent the mean +/- SD of three determinations. Luciferase activity is reported as relative light units (RLU) normalized to the protein concentration of BG1Luc4E2 cell lysate using the fluorescamine assay (see Materials and Methods). Dilutions of Surflan™ (1/10,000 and 1/100,000) were significantly different than controls ($p = 0.002$, 0.02 respectively; t-Test).

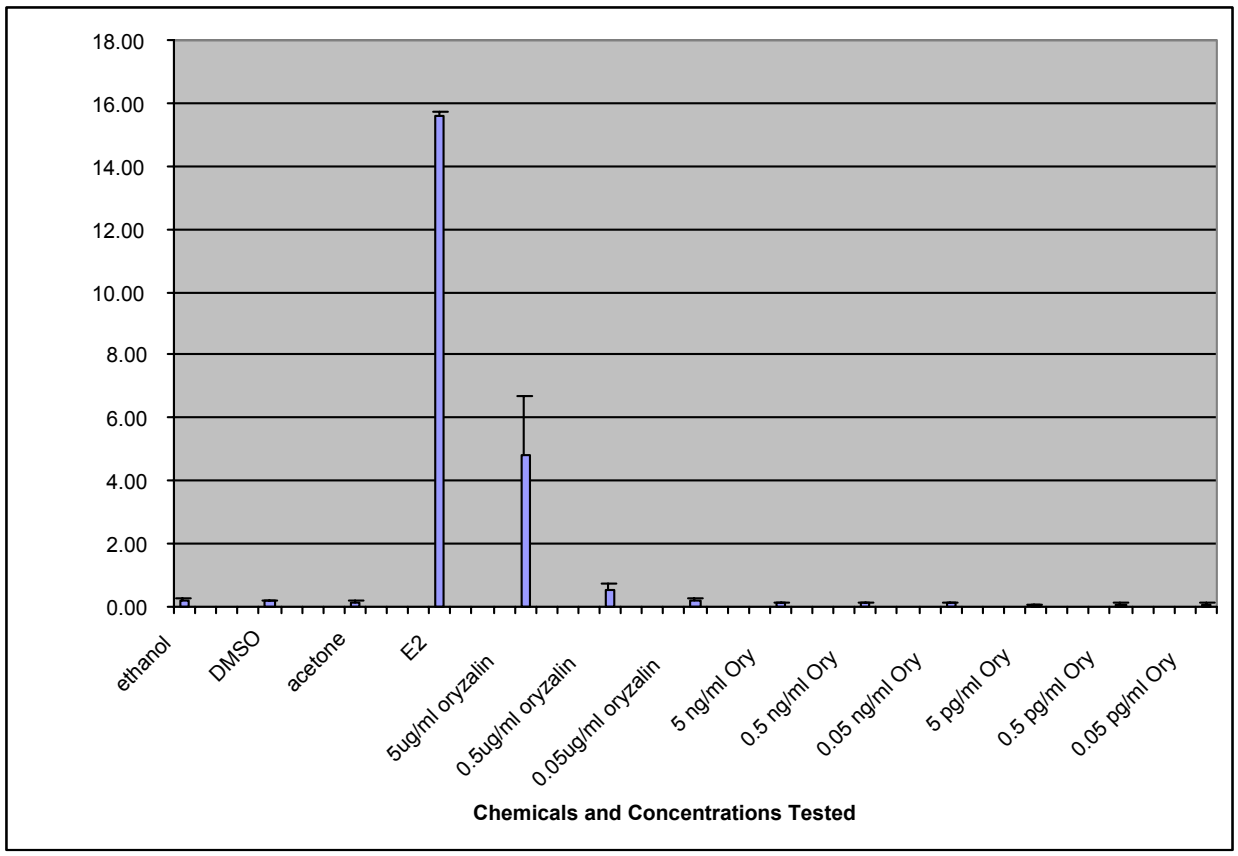

Figure 1-2. Initial Screening of oryzalin in BG1Luc4E2 Cells. Values represent the mean +/- SD of four determinations. Luciferase activity is reported as relative light units (RLU) normalized to the protein concentration of BG1Luc4E2 cell lysate using the fluorescamine assay (see Materials and Methods). The response of oryzalin tested at 5 µg/ml and 0.5 µg/ml was significantly different from DMSO controls ($p = 0.02$, t-Test).

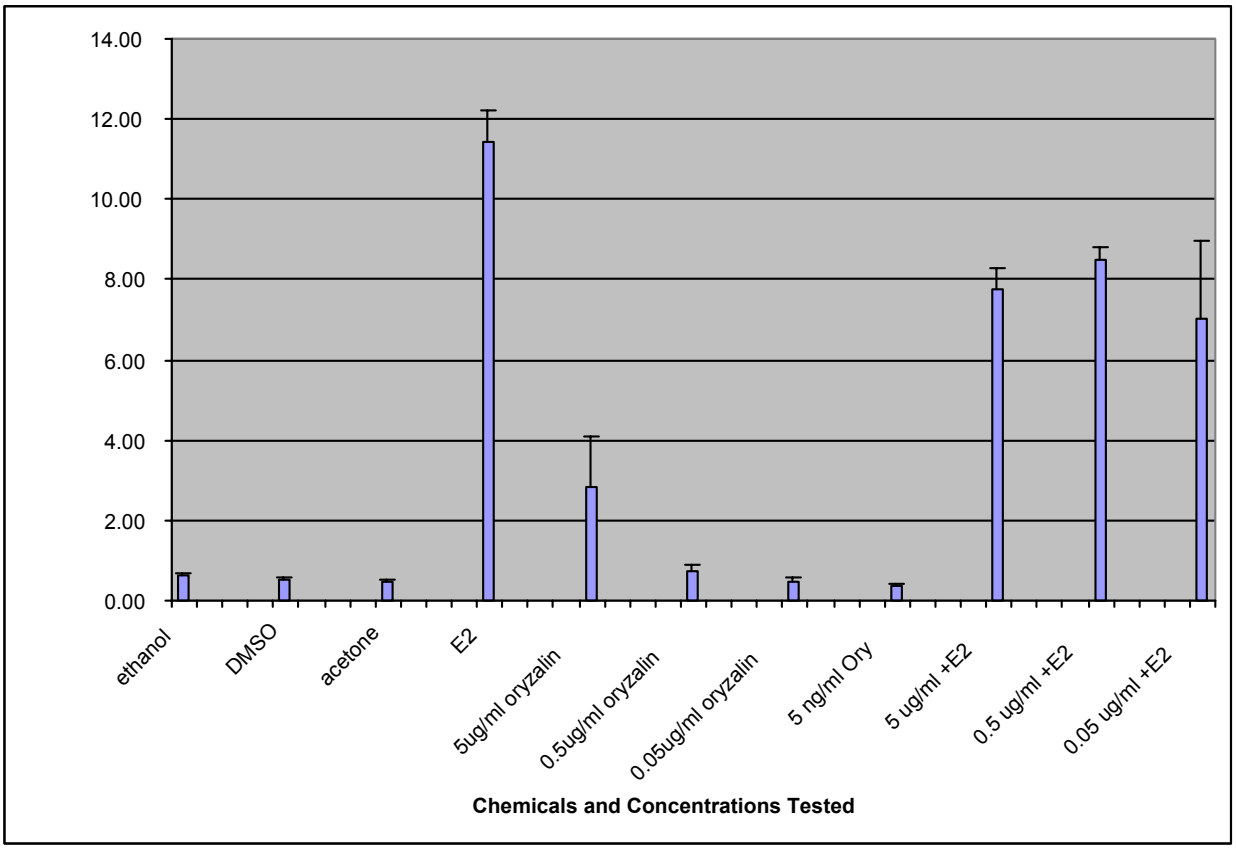

Figure 1-3. Estrogenic Effects of oryzalin in BG1Luc4E2 Cells. Values represent the mean +/- SD of four determinations. Luciferase activity is reported as relative light units (RLU) normalized to the protein concentration of BG1Luc4E2 cell lysate using the fluorescamine assay (see Materials and Methods).). The response of oryzalin tested at 5 µg/ml and 0.5 µg/ml was significantly different from DMSO controls ($p = 0.03$, $p = 0.04$, respectively t-Test). The responses of oryzalin (5, 0.5, and 0.05 µg/ml) co-incubated with 1 nM estradiol were all significantly different than DMSO controls ($p < 0.01$, t-Test).

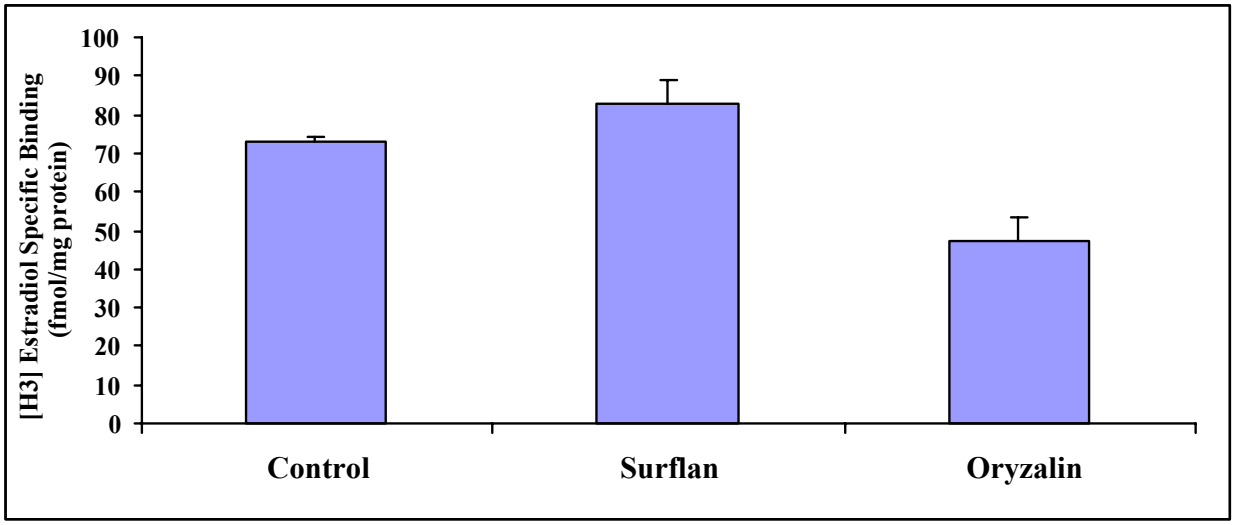

Figure 1-4. Specific Binding of Surflan™ (1/10,000 dilution) and oryzalin (100 µM) to Calf Uterine Cytosol Estrogen Receptor. Data represent mean +/- SD of three determinations. The displacement of [H3] estradiol by oryzalin was significant ($p = 0.01$, t-Test).

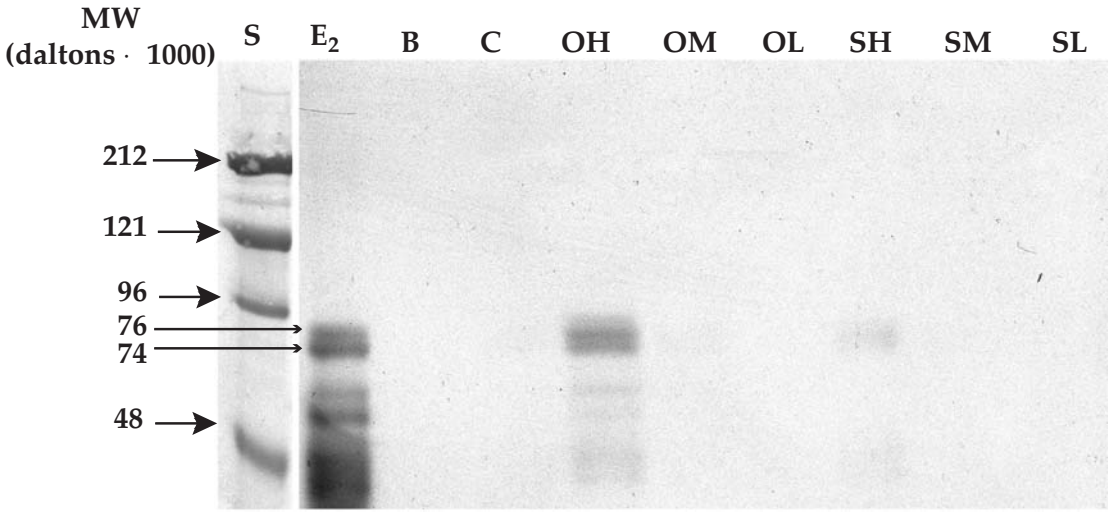

Figure 1-5. Western blot of male medaka liver proteins separated on a 10% Tris-HCl gel, reacted with polyclonal rabbit anti-medaka choriogenin antibody and visualized with a goat anti-rabbit IgG alkaline phosphatase conjugate secondary antibody. Column S shows molecular weight (MW) standards. Numbered arrows give the MW of the individual protein standards and of the choriogenin bands at 76,000 and 74,000 daltons. E2 (17β-estradiol, 15 ng/ml); B (blank); C (control); OH (oryzalin high-dose, 3.3 mg/l); OM (oryzalin mid-dose, 2.2 mg/l);OL (oryzalin low-dose, 1.5 mg/l); SH (Surflan™ high-dose, 8.5 µl/l); SM (Surflan™ mid-dose, 5.5 µl/l); SL(Surflan™ low-dose, 3.8 µl/l). The figure demonstrates that E2, OH and SH induced expression of chroiogenins.

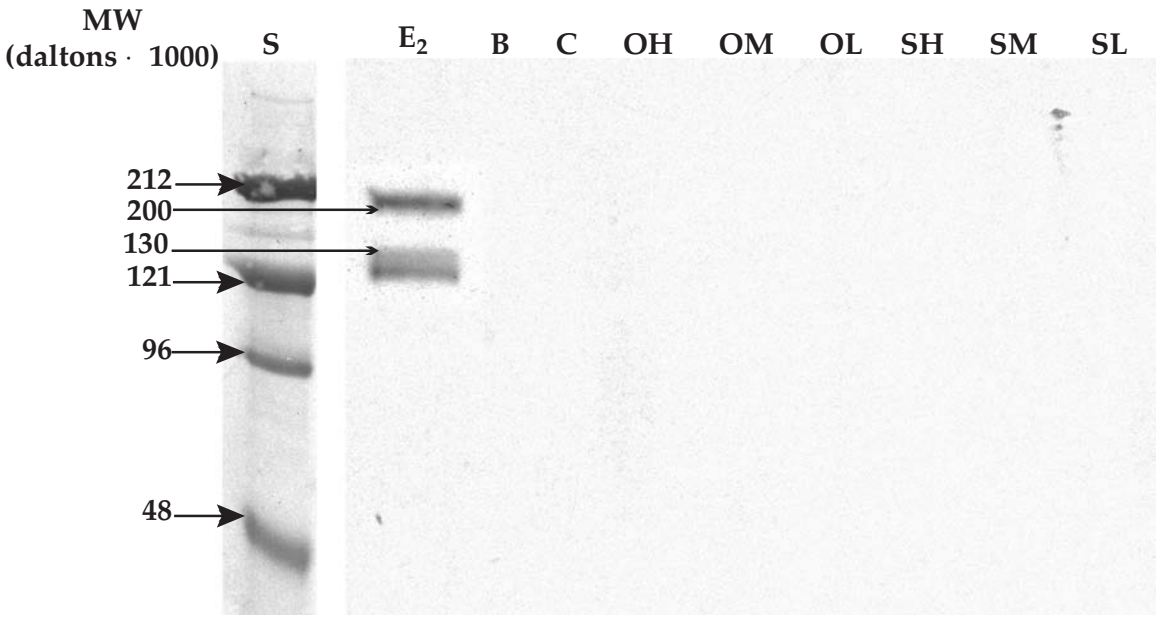

Figure 1-6. Western blot of male medaka liver proteins separated on a 10% Tris-HCl gel, reacted with mouse anti-striped bass vitellogenin and visualized with a goat anti-mouse IgG alkaline phosphatase conjugate secondary antibody. The figure demonstrates that only E2 (17β-estradiol) induced expression of vitellogenin. Column S shows molecular weight (MW) standards. Numbered arrows give the MW of the individual protein standards and of the vitellogenin bands at 200,000 and 130,000 daltons. E2 (15 ng/ml); B (blank); C (control); OH (oryzalin high-dose, 3.3 mg/l); OM (oryzalin mid-dose, 2.2 mg/l); OL (oryzalin low-dose, 1.5 mg/l); SH (Surflan™ high-dose, 8.5 µl/l); SM (Surflan™ mid-dose, 5.5 µl/l); SL (Surflan™ low-dose, 3.8 µl/l).

SECTION II

REPRODUCTIVE TOXICITY OF ORYZALIN AND SURFLAN™ TO MALE AND FEMALE JAPANESE MEDAKA

Surflan™ is a proprietary herbicide emulsion formulated and marketed by Dow Elanco. Oryzalin (3,5-dinitro-N4, N4-dipropylsulfanilamide) is the active ingredient of Surflan™, comprising 40.4% by weight. In Section 1, we identified both Surflan™ and oryzalin as xenoestrogens – compounds that may be capable of eliciting many of the biological responses of endogenous estradiol.

In teleosts, endogenous estrogens (primarily 17β-estradiol) have a multitude of functions in normal physiology, and are involved in sexual differentiation of embryos (Bye and Lincoln, 1986; Piferrer and Donaldson, 1989), sexual maturation (Yamamoto, 1969; Goetz et al., 1979; Van den Hurk et al. 1982), and reproductive behavior (Hutchinson, 1993). Long-considered to be principally a female reproductive hormone due to its roles in vitellogenesis and choriogenesis (Chester-Jones et al., 1987), the identification of estrogen receptors (ERs) in brain and reproductive tissues of male vertebrates (Kato et al., 1974; Murphy et al., 1980; Mak et al., 1983a,b; Callard and Mak, 1985; Cooke et al., 1991) point to estrogens' importance in male reproduction as well.

Pituitary gonadotropin hormones (GtH) untimately control reproduction. Two discrete GtHs have been characterized in teleosts — GtH-1 and GtH-II — which correspond to the mammalian gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), respectively. The gonadotropins act by regulating the synthesis of the principal gonadal steroid hormones

testosterone and estradiol, which in turn, control gonadal maturation and gametogenesis (Redding and Patino, 1993).

Estradiol is synthesized in the brain, ovaries, and testes by the action of cytochrome p450 (p450arom), which converts testosterone to estradiol (for reviews see Simpson et al., 1994; Conley and Hinshelwood, 2001). In the gonads, p450arom activity is regulated by the gonadotropins, which in turn are subject to feedback regulation by testosterone and estradiol (Nagahama, 1987). As the primary substrate for p450arom and a metabolic precursor to estradiol, testosterone levels are regulated both by estradiols' action on the gonadotropins and on 17-α hydroxylase, C17/20 lyase–a key enzyme in the biosynthesis of testosterone (Dufau, 1988; Majdic et al., 1996). In the brain, where ERs and p450arom tend to be co-located in reproductively important regions, conversion of testosterone to estradiol is crucial for mammalian male reproductive behavior (reviewed in McEwen et al., 1979). In at least some species of teleosts, brain p450arom expression may be subject to regulation by estradiol (Callard et al., 2001).

Estrogen homeostasis is critical to the normal function of male and female gonads, to gametogenesis, and to reproductive behavior. Exogenous estrogen and xenoestrogens appear capable of disrupting this homeostasis; administration of exogenous estradiol, the pharmaceutical estrogen 17α-ethynylestradiol (17α-EE2), or various xenoestrogens has been correlated with adverse reproductive effects in fish including decrements in fertility (Gray et al., 1999b; Gronen et al. 1999; Van den Belt et al., 2001); testicular degeneration and atrophy (Jobling et al., 1996; Christiansen et al., 1998; Miles-Richardson et al., 1999); inhibition of spermatogenesis (Billard

et al., 1981; Jobling et al., 1996; Kinnbert et al., 2000; Sohoni et al., 2001); inhibition of testicular development (Gimeno et al., 1998; Panter et al., 1998); development of intersex testes (Gray et al., 1999a; Koger et al., 2000; Metcalfe et al., 2000; Yokota et al., 2001); and ovarian degeneration or suppressed ovarian development (Lange et al., 2001; Van den Belt et al., 2001). In general, the physiological alterations underlying these effects have not been identified. However, certain studies have implicated administration of exogenous estradiol or xenoestrogens in the suppression of gonadotropins (Lessman and Habibi, 1987; Harris et al., 2001), adverse effects on reproductive behavior (Gray et al., 1999b; Gronen et al., 1999; Bjerselius et al., 2001; Van den Belt et al., 2001); and alterations in p450arom activity (Melo and Ramsdell, 2001) or expression (Scholz and Gutzeit, 2000).

Medaka (*Oryzias latipes*) are commonly chosen as a model organism for use in reproductive studies due to their high, relatively consistent, and reproducible reproductive capacity; under specific culture conditions of photoperiod (16h light: 8h dark), water temperature (25°C ± 1°C; and regular feeding (3-4% of body weight per day), reproductively mature females may produce upwards of 7000 eggs per year (Kirchen and West, 1976). Egg production occurs at dawn under natural light, and can be controlled by photoperiod such that females produce eggs approximately daily on a year-round basis (Kirchen and West, 1976; Koger et al., 1999). Medaka eggs are produced in clusters of 1-70, but most typically in groups of 10-25; these eggs develop externally. Their transparent chorion allows simple visual determination of fertilization, detectable within 0.2 h by the presence of yolk and a perivitelline space. Medaka larvae hatch within 1-3 weeks, depending on the specific culture conditions (Kirchen and West, 1976).

The demonstrated ability of many xenoestrogens to impair reproduction indicated that Surflan™ and oryzalin may have similar activity. As newly identified xenoestrogens, it is important to characterize the effects of these substances on indices of reproductive fitness such as fertility and fecundity; measurement of these parameters can give the first indication of the likelihood that effects of exposure may extend beyond the individual and potentially impact a population. Accordingly, the present study was designed to determine whether exposure of reproductively active, mature male and female medaka to Surflan™ and oryzalin would affect fertilization success or fecundity; viability of eggs or time-to-hatch; or cause deformities in the offspring. We also evaluated gonadal tissues of males and females in order to identify and characterize any exposure-related effects.

Materials and Methods

Animals

The medaka used in this study were the golden strain of *Oryzias latipes*, originally purchased from Carolina Biological Supply. The colony was established at the University of California, Davis (UCD) in 1986, and has been maintained at the UCD Aquatic Center since that time. The colony's brood stock has been out-crossed twice since 1986 with wild-type Northern strain golden medaka to prevent inbreeding (Davis, 2001).

Reproductive effects study design

168 sexually mature, five month-old medaka were separated into six replicate groups for each treatment, with two female and two male fish per replicate. Only reproductively active females were utilized, and were identified by the presence of an attached, fertilized clutch of eggs on the day of collection. Each group of four medaka was placed in an aerated 1-l glass beaker of

moderately hard U.S. EPA reconstituted water (Horning and Weber, 1985), maintained at approximately 25 $\pm$ 1°C. A photoperiod of 16h light, 8h dark was maintained throughout the experiment, with light provided at 400-450 lux by Vita-Lite (Coralife Chromatic™) bulbs.

Final exposure concentrations of Surflan™ and oryzalin (both from Dow Elanco) were selected from the results of a preliminary study in which 168 medaka were exposed for 21d to nominal concentrations of Surflan™ (8.5, 5.8, or 3.8 ul/l), or oryzalin (2.0, 1.3, or 1.0 mg/l). Concentrations for the high-dose group of each chemical were selected as the 21-day maximum tolerated concentration (MTC), where tolerance was considered to be those concentrations associated with 15% lethality or less during the exposure period. Exposure concentrations for each of the mid- and low-dose groups were set at approximately half of the concentration of each higher dose.

Exposure to nominal concentrations of oryzalin (1.0, 0.5, or 0.25 mg/l) or Surflan™ (3.8, 2.0, or 1.0 µl/l) or reconstituted water was initiated after fish had been allowed to acclimate for five days, and it had been established that each replicate group was actively producing eggs. Exposure solutions were made fresh daily, and the entire solution of each beaker was replaced every day for the 21-d exposure period. On a daily basis, fish were fed a purified casein (PC)-based diet (DeKoven et al., 1992) at 2% of their body weight prior to solution change, then received brine shrimp at approximately 3.5% of body weight after solution replacement.

At the end of the 21-d exposure period, fish from each treatment were separated by exposure group and gender. Two untreated males were placed with each pair of treated females, and two

untreated (reproductively active) females placed with each pair of treated males. Newly-introduced fish were of the same broodstock and hatch date as the treated animals. Each new group of four medaka was placed in a fresh 1-l beaker, and maintained in reconstituted water (Horning and Weber, 1985) for the remainder of the experiment. The entire 1-l volume of water in each beaker was replaced daily throughout the remainder of the experiment. All fish received the same diet as described above.

Following the placement of untreated medaka with treated medaka, fish were allowed to acclimate for two days. Egg collection began on day three post-exposure, and continued for five days thereafter. Eggs were siphoned twice daily from each beaker for five days, counted, and evaluated visually under a microscope to determine fertilization status. Egg masses were separated with forceps to obtain individual eggs, and unfertilized eggs were counted, recorded and discarded. For the first three days of egg collection, fertilized eggs from each replicate were counted and transferred to a 600-mL aerated beaker and observed daily for mortality and hatch. Beakers were maintained in a water bath of approximately 25°C and a photoperiod of 16h light:8h dark. To minimize fungal growth, embryos that died were counted and removed from beakers daily. Approximately 75% of the water was exchanged daily from each beaker. Beakers were observed on a daily basis to detect the presence of newly hatched larvae. Larvae were counted and the date of hatch noted. Larvae were transferred to an aerated 40-l aerated tank for grow-out (one tank per treatment group). The water temperature and photoperiod were maintained as previously described. Upon transfer to a tank for grow-out, larvae were fed a PC diet graded to the appropriate size. Larvae were raised until approximately 30 days of age, at

which time a sample of 50 juvenile fish from each tank were sacrificed and examined visually for the presence of any gross abnormalities.

Histologic effects study design

Eighty-four, seven month-old sexually mature medaka were separated into three replicate groups for each treatment, with two male and two female fish per replicate. Each group (n = 4) was placed in an aerated 1-l beaker of reconstituted water, and maintained as described above. Following a two-day acclimation period, exposures to Surflan™ and oryzalin were initiated. Exposure concentration, solution preparation, and solution exchange procedures were the same as those used in the reproductive effects study (see above).

At the end of the 21-d exposure period, medaka were euthanized by immersion in tricaine methane sulfonate (MS222, Sigma) and examined grossly. Abdominal cavities were surgically opened, and fish were immediately immersed in 10% neutral buffered formalin. Formalin-preserved fish were processed with standard methods for paraffin embedding. Two initial sections were obtained from each paraffin block, mounted on glass slides and stained with hematoxylin and eosin (HE). If representative sections of gonads were not obtained during the initial processing, paraffin blocks of fish were step-sectioned (at 50 μm intervals) for an additional four to six levels as needed to obtain adequate gonadal views.

Histologic methods

Glass HE slides were initially sorted by gender and then randomized prior to reading and scoring. All slides were read and scored blind (no knowledge of exposure history). Slides were initially screened for lesions in gonads, followed by selection of lesions to score, and

development of scoring spreadsheets. Lesions were scored semi-quantitatively: 0 = not present; 1 = mild; 2 = moderate; 3 = severe. An average lesion score was determined for each lesion type and for each exposure group. Criteria for determining lesion scores were different for males and females because of normal sexual dimorphism in organ histo-morphology. In general, scoring criteria were based on the number and size of lesions within a set field or organ. The "fields" referred to in the scoring criteria descriptions refer to microscope objective fields. For example, a 40x field refers to a 40x microscope objective field, which gives a total magnification of 400x (40x objective times 10x ocular magnification). For the majority of testicular lesions, lesion score criteria are based on a "half 40x field." A "half" field was used because most testicular lesions occurred in seminiferous tubules in the cortical (subcapsular) region of the testes, and lesions were evaluated by scanning the testes with only half the 40x field in use.

Table 2-1 identifies testicular lesion types and lesion scoring criteria for male medaka. Pre-intersex lesions (PIL) are histologic alterations that appeared to precede overt intersex changes (oocyte formation) in testes of male fish. PILs consisted of enlargement of individual spermatogonia within seminiferous tubules at the periphery of the testis (usually adjacent to the capsule). In PILs, hypertrophied spermatogonia were two to four times larger than normal, with karyomegaly and prominent single nucleoli. The most obvious transitional cells were those with marked karyomegaly where the chromatin cleared (indicative of increased euchromatin) and contained numerous provitelline nucleoli, characteristic of those found in immature oocytes.

Evidence of overt intersex change was observed as the presence of immature primary oocytes in testicular tissue; these testes were classified as having intersex lesions with stage 1 oocytes (S1-

Oo). These earliest intersex lesions appeared as clusters of small cells, usually arranged in the form of discrete acini, that were distinct (and often artifactually separated) from normal seminiferous tubules. These clusters of small cells were usually deep within the testicular parenchyma, below the subcapsular layer of seminiferous tubules, and were characterized by: 1) differences in tinctorial staining (pale eosinophilic to amphophilic cytoplasm and more refractile than normal spermatogonia); 2) a higher nucleus to cytoplasm ratio; and 3) prominent oval nuclei with more euchromatin and the absence of distinct nucleoli. These cells were classified as stage 1 oocytes, and their occurrence in small clusters or acini was similar to oogonial nests found in immature ovaries. Oogonial nests in medaka testes contained anywhere from 3 to 25 small cells.

The most obvious evidence of intersex change in male medaka testes was the appearance of stage 2 and stage 3 oocytes (S2/3-Oo). Stage 2 oocytes were characterized by the presence of large central nuclei with diffuse euchromatin and small amounts of intensely basophilic cytoplasm. Stage 2 oocytes varied in size from 5-15 μm and were usually solitary. Occasionally, stage 2 oocytes contained characteristic larger nuclei, but did not have basophilic cytoplasm. Stage 3 oocytes were large, typically containing 15 to 45 cells characterized by abundant, intensely basophilic (blue-purple) cytoplasm and marked karyomegaly (nuclei ranging from 4 to 30 μm) with translucent euchromatin and provitelline nucleoli. Stage 3 oocytes were often found in clusters of up to 30 μm.

The level of sperm production and/or depletion was evaluated in multiple sections of testes, with the primary focus on the central portion of the organ where mature spermatozoa are stored.

Testicular sperm depletion (TSD) was characterized by smaller than normal seminiferous tubules with fewer spermatids and mature sperm. Testes with severe sperm depletion had tubules that were only partially filled and had a patchy appearance. Necrosis of spermatids (NST) was a lesion characterized by spermatids with large (2-5 times normal), irregular, hyperchromatic heads. Necrosis of spermatogonia (NSG) was a relatively common lesion characterized by rounding up of individual cells, hyper-eosinophilic cytoplasm, and nuclear pyknosis. Necrotic spermatogonia were most often found individually, but in some cases were observed in small clusters.

Ovarian Macrophage Aggregate (OMA) was a common finding in medaka ovaries (Table 2-2). OMA's were composed of large macrophages filled with foamy, pale eosinophilic debris, and were primarily concentrated in the central (medullary) portion of the ovary. However, severely affected fish had macrophage aggregates that were scattered throughout the ovary. Aggregates varied widely in size and shape. A minimum average diameter of 50 μm was designated as the cutoff point for inclusion as a lesion. Larger aggregates were subdivided by 50 μm to normalize lesion scoring. To further normalize scoring, the average number of aggregates was determined by counting the two best 20x fields on a slide.

Ovarian Follicular Necrosis (OFN) was identified by the presence of necrotic follicles that were large (several hundred microns in diameter), collapsed, distorted from the normal spherical shape, and empty – i.e., there was no developing oocyte within the follicle. Affected follicles were lined by necrotic columnar epithelium, and dead cells were characterized by marked nuclear pyknosis and karyorrhexis. Follicular necrosis was distinct from oocyte atresia in that

atretic follicles involved large developing oocytes, while follicular necrosis involved "empty" follicles.

Oocyte Atresia (OA) was characterized by atretic oocytes or follicles that were usually large and well developed, with distinct chorions and abundant yolk-protein. Atretic oocytes were distinguished by distortion and collapse of the chorion, fragmentation and disorganization of the yolk and oil droplets, and variable influx of macrophages.

Ovarian Hypertrophy / Hyperplasia (OHH) was differentiated by overall enlargement of the ovary and an increased number and density of developing oocytes. OHH was scored in female medaka based on the size of the ovary in comparison to size of the peritoneal cavity. OHH is not a lesion; it is the manifestation of normal, cyclical transformations that the ovary undergoes in preparation for egg production. In wild mature medaka, OHH is probably seasonal and dependent on water temperature, light intensity, and photoperiod. In immature medaka, OHH is a component of normal development as the ovary increases in size because of enlargement of developing oocytes. However, estrogen is capable of affecting ovarian development (see discussion), and changes in OHH severity were evaluated as a potential indicator of estrogenic effect. Similarly, immature oocytes (IO) are not lesions per se, as they represent a normal stage of oocyte development. IO's were assessed by applying a scoring system to the percentage of oocytes within an ovary classified as immature. These immature oocytes were characterized by: 1) small size; 2) an absence of a definitive chorion; 3) the absence of distinct yolk protein or oil droplets; 4) by having abundant basophilic cytoplasm; and 5) having discrete nuclei.

Statistical analyses

Fertility and fecundity data and both testicular and ovarian lesion scores were evaluated by applying the nonparametric Kruskal Wallis test for one-way ANOVA using NCCS or JMP statistical software. When comparisons were made on the basis of dose group, α values were adjusted by a Bonferroni correction to account for multiple comparisons, such that $p < 0.008$ represents a result that is significantly different from controls. Adjustments of α values were not made for testicular and ovarian lesion-scores evaluated by treatment category, and an outcome of $p < 0.05$ was identified as statistically significant. Time-to-hatch data were tested for statistical significance by application of the Wald-Wolfowitz Runs Test, where a result of $p < 0.05$ represented statistical significance. The daily number of eggs hatched for each treatment group was used to develop cumulative probability distributions of the percentage of eggs hatched for each day of the hatching period. These distributions were compared by the Wald-Wolfowitz Runs Test, which tests the hypothesis that any two samples (i.e., distributions) came from populations with identical distribution functions. A statistic — the total number of runs — is computed for each pair of samples, where a run consists of a sequence of consecutive ordered values from one population, compared to a comparable ordered sequence from the population of interest. Each sample comparison also provides information on the sign i.e., negative or positive, of the comparison outcome. In our application of this test, the sign of the outcome indicated whether the time-to-hatch was increased or decreased by treatment.

Results

Exposure to Surflan™ or oryzalin for 21d affected the reproductive outcomes of medaka, and caused detrimental effects on fertility and on the incidence and/or severity of testicular and

ovarian lesions. Surflan™ and oryzalin also significantly delayed the hatching time of eggs produced by treated males paired with untreated females, and significantly shortened the hatching time of eggs produced by treated females.

The daily mean production of non-fertilized (NF) eggs by treated females paired with untreated males, and by untreated females paired with treated males was consistently greater than controls in all dose groups (Table 2-3). Low-dose oryzalin females, mid-dose oryzalin males, and mid-dose Surflan™ males had significantly greater daily mean production of NF eggs than controls; additionally, the daily mean production of NF eggs approached significance in mid-dose oryzalin females. Considering treated females and males together (F+M), the daily total production of NF eggs was significantly greater than in controls in the mid-dose Surflan™ group, as well as in the mid- and low-dose oryzalin groups.

The mean daily total production of eggs was not uniformly affected by exposure to Surflan™ or oryzalin (Table 2-4). Although several treatment groups produced lower total numbers of eggs than controls, none of these apparent effects reached statistically significant levels. However, results approached statistical significance in low-dose oryzalin males, high-dose Surflan™ females, and the corresponding dose groups when F+M were considered together.

Viability of fertilized eggs was not affected by treatment, with a mean hatch rate typically $\geq$ 90% (Table 2-5).

The results of the Wald-Wolfowitz Runs Test (Table 2-7) demonstrate that the time-to-hatch in all Surflan™ and oryzalin treatment groups was significantly different from controls. In eggs produced by untreated females paired with treated males, hatching time was significantly delayed relative to controls. The opposite trend was apparent in eggs produced by treated females paired with untreated males, where time-to-hatch occurred significantly earlier than in eggs produced by controls.

A very low overall incidence of gross deformities was observed in juveniles sampled at approximately 30-d post-hatch (< 5 total deformed juvenile fish); there were no treatment-related effects (data not shown).

A 21-d exposure to Surflan™ or oryzalin induced intersex lesions with S1-Oo and/or S2/3-Oo's in male medaka in all treatment groups (Table 2-8). PIL were also observed in all treatment groups, and there was a low but measurable occurrence of PIL in controls. One control male exhibited a S1-Oo lesion (see discussion). There was a marked difference in prevalence of S1-Oo's, S2/3-Oo's, or total intersex lesions (S1-Oo or S2/3-Oo) between treated and control groups, where prevalence refers to the number of animals in a treatment group to manifest a specific intersex lesion(s). Surflan™ appeared to be more effective than oryzalin in inducing intersex, in that all doses of Surflan™ caused higher total intersex prevalence than any of the oryzalin dose groups. Total intersex prevalence approached statistical significance in the two highest Surflan™ dose groups, and was clearly significantly different from controls in the low dose Surflan™ group, where total intersex prevalence reached 100 %. Total intersex prevalence was not significantly different from controls in any of the oryzalin groups, despite the fact that

prevalence ranged from 50 to 67%. Intersex prevalence data by treatment category (control, oryzalin, or Surflan™) are presented in Figure 2-1.

The mean of the testicular lesion scores for PIL was greater, but not significantly different from controls, in all treatment groups (Table 2-7). The mean lesion scores for S1-Oo's were also greater than controls in all treatment groups; mean lesion scores were statistically significant only in males exposed to low-dose Surflan™, although the mean score in the high-dose Surflan™ group neared statistical significance. No stage S2/3-Oo's were detected in control testes, but were detected in one or more animals in each of the groups exposed to Surflan™ or oryzalin. The mean severity score for stage S2/3-Oo's approached statistical significance only in high-dose Surflan™ animals. When the mean severity scores for stage S1-Oo and S2/3-Oo's combined were calculated, all Surflan™ dose groups as well as the mid-dose oryzalin group exhibited scores that neared statistical significance. Intersex lesion scores analyzed by treatment category (i.e., exposure to Surflan™ or oryzalin) showed that exposure to Surflan™ , but not oryzalin, induced a statistically significant increase in the mean lesion severity score for PIL, S1-Oo, and S2/3-Oo evaluated together , and for S1-Oo and S2/3-Oo combined (Table 2-8). Figure 2-2 represents a comparison of average intersex lesion severity scores for S1-Oo's, S2/3-Oo's, or S1-Oo and S2/3-Oo's combined, compared by treatment category.

Some NST and NSG were observed in control animals (Table 2-7), although the mean severity scores for these lesions were generally higher in animals exposed to Surflan™ or oryzalin. The mean lesion severity score for NST approached statistical significance in mid and low dose oryzalin animals; mean lesion severity scores for NSG approached statistical significance in the

mid dose Surflan™ and mid dose oryzalin groups, and was significantly different from controls in low dose oryzalin animals. The combined mean lesion score for NST and NSG was significantly different from controls in the mid dose oryzalin group. Evaluation of NST, NSG, and NST and NSG combined by treatment category demonstrated that oryzalin but not Surflan™ caused statistically significant increases in the mean lesion severity score for all three categories of testicular lesion (Table 2-8). TSD occurred in animals from all groups, and there was no discernable effect of treatment (Table 2-7).

Two categories of ovarian lesion – OA and OFN– showed little or no effect of exposure to either Surflan™ or oryzalin (Table 2-9). Additionally, the mean lesion severity scores of OMA were greater than controls in four of six treatment groups, but only approached statistical significance in females in the low dose Surflan™ group.

All but one exposure group (mid dose Surflan™) exhibited mean lesion scores for IO's that were lower than controls. This apparent effect of treatment was especially marked in animals exposed to oryzalin; all oryzalin dose groups exhibited mean lesion scores for IO's that were less than half those determined for controls. None of these results was statistically significant when evaluated on the basis of dose. However, when the mean severity scores of IO's were evaluated by treatment category i.e., exposure to oryzalin or exposure to Surflan™, the mean lesion severity scores for IO's in oryzalin-exposed animals was significantly lower than in controls (Table 2-10). The severity of OHH also seemed to be affected by exposure to Surflan™ and oryzalin, with mean lesion severity scores in all treatment groups greater than those in controls (Table 2-9). All oryzalin dose groups and the low-dose Surflan™ group had mean lesion

severity scores for OHH that approached statistical significance. Evaluation of OHH by treatment category (Table 2-10) demonstrated that exposure to oryzalin was associated with a statistically significant higher mean lesion score for OHH relative to controls.

In general, the occurrence of gonadal lesions of each category in control animals, combined with small sample size (n = 5 or 6), a limited number of numerical response possibilities (0,1, 2, or 3), and application of Bonferroni's correction for multiple comparisons made it very difficult to demonstrate that an apparent effect of treatment was statistically significant.

Discussion

Male medaka undergo sexual differentiation of the gonad at approximately 2 weeks post-hatch (Okada, 1964). Induction of intersex lesions in genotypic adult male medaka with differentiated testes following exposure to estradiol or xenoestrogens has been reported by Egami (1955), Shibata and Hamaguchi (1988), and Gray et al. (1999a) among others. Induction of intersex in juvenile medaka is described by Gray and Metcalfe (1997), Koger et al. (2000), Metcalfe et al. (2000), Lange et al. (2001) and Yokota et al. (2001). The gonadal plasticity exhibited by male medaka has been attributed to the existence of bipotential spermatogonia, whereby type B (secondary) spermatogonia retain the ability to form ovarian tissue under estrogenic influences (Shibata and Hamaguchi, 1988). The bipotentiality of spermatogonia is retained even following intersex-inducing estradiol treatment, as intersex testes appear to be reversible to normal testes subsequent to the withdrawal of the estrogenic influence (Shibata and Hamaguchi, 1988).

In the present study, intersex was induced in 10/18 (55.6%) of medaka exposed to oryzalin and in 15/17 (88%) of medaka exposed to Surflan™ for 21d. Total intersex prevalence evaluated by dose group ranged from 80 to 100% after treatment with Surflan™, and 50 to 67% after treatment with oryzalin. These are comparatively high incidences relative to the 17% incidence reported by Gray et al. (1999a) for adult medaka exposed to 200 or 300 µg/l octylphenol for 18 or 36d. The intersex incidence induced by Surflan™ or oryzalin are more comparable to the ~ 70% incidence reported by Egami (1955) in medaka exposed to 80 or 160 µg/l estradiol for 31 to 37d. [Shibata and Hamaguchi (1988) did not report their data in terms of incidence.] Because the relative estrogenic potency of Surflan™ and oryzalin have not been characterized, direct comparisons between intersex induction rates in medaka by Surflan™ or oryzalin to intersex rates induced by estradiol or octylphenol cannot be made. It does appear however, that both Surflan™ and oryzalin are relatively effective at inducing intersex lesions.

The biological significance of intersex lesions to an individual fish is unclear, in part because intersex lesions tend to regress once the estrogenic stimulus is withdrawn (Okada, 1964; Shibata and Hamaguchi, 1988). Furthermore, it is not clear whether intersex lesions within a testis affect fertility. Gray et al. (1999b) reported that a male medaka exposed from 1d posthatch to 6 months of age, and then allowed to mate with an unexposed female, was able to fertilize eggs; that same male was later found to have an intersex testis. Additionally, the administration of estradiol or xenoestrogens to experimentally-induce intersex can have separate adverse effects on other aspects of testicular morphology and spermatogenesis (see following), and consequently, it is difficult to attribute decrements in reproductive performance to the occurrence of a single type of lesion.

The spontaneous development of intersex testes in medaka has not been formally reported in the open literature. However, the experience in our laboratory has been that intersex testes do occur – albeit very infrequently– in naïve fish (Koger et al., 2000; Davis, 2002). One possible explanation for the occurrence of intersex testis in one of the control males is that these lesions can be induced by a potentially estrogenic compound present in the PC diet used by our laboratory. The PC diet that we formulate and prepare contains soy lecithin at approximately 6% of the total dry weight. Soy food products are estrogenic in humans (for ex. see Kurzer, 2002), and it is likely that they would have similar activity in teleosts. However, we do not know if soy-derived lecithin is estrogenic, or if it is, whether daily ingestion and/or absorption across the gills after dissolution of diet in aquaria water are capable of inducing intersex in medaka. Alternatively, estrogens released by females into aquaria water may be able to induce intersex– either singly, or in combination with the soy-derived lecithin. That biologically active levels of estrogen may occur in aquaria water is indicated by reports of vitellogenin (Vg) in unexposed male fish (Tyler et al., 1996; Panter et al., 1998; Tyler et al., 1999). Vg expression is regulated by estradiol, and typically, is not produced at measurable levels in males in the absence of estrogenic stimulus (see Section 1 for discussion). We do not know that biologically active levels of estrogen are present in aquaria water in our facilities however, nor do we know whether levels of estrogen, if present, are sufficient to induce intersex testes. Nonetheless, although we cannot definitively explain the occurrence of the S1-Oo lesion in a control male, it is clear that both Surflan™ and oryzalin dramatically affect the occurrence and severity of intersex lesions in medaka.

Successful reproduction in male teleosts depends on hormonal homeostasis of estradiol, androgens, and the gonadotropins (Nagahama, 1987; Trudeau, 1997). Supression of GtH levels in mammals by hypophysectomy, immunoneutralization, administration of the xenoestrogen octylphenol, or the estrogen agonist diethylstilbestrol (DES) have led to germ cell degeneration, lowered testosterone secretion, decreased sperm numbers and testis weight, and inhibited spermatogenesis (Russell and Clermont, 1976; Raj and Dym, 1976; Bartlett et al., 1989; Nonclerq et al., 1996; Blake and Boockfor, 1997; Boockfor and Blake, 1997). DES also caused a 10-50-fold increase in testicular cell apoptosis, with the magnitude of effect depending on the duration of exposure (Nonclerq et al., 1996). Gonadal production of estradiol is also critical to normal gametogenesis; in mice that lack a functional aromatase gene, fertility deteriorated with age due to increasing impairment of spermatogenesis even though gonadotropin and androgen levels were not affected (Robertson et al., 1999).

In teleosts, endogenous estradiol can have either negative or positive feedback 1993). The latter action may occur either via negative feedback effects at the pituitary, or potentially by direct action on the testis to inhibit testoterone synthesis by the 17-α hydroxylase, C17/20 lyase complex (Dufau, 1988; Majdic et al., 1996).

In this study, Surflan™ and oryzalin adversely affected spermatogenesis, increasing the incidence and severity of NST and NSG in treated animals above that observed in controls. In mammals, germ cell degeneration is a common phenomenon in adult males (Huchkins, 1978; Kerr, 1992), and Nonclerq et al. (1996) documented a low but measurable incidence of apoptosis of cells in the seminiferous epithelium of untreated male hamsters. Although we are not aware

of analogous data specific to teleosts, it is likely that a similar phenomenon occurs and is the underlying basis for the occurrence of NST and NSG in controls.

The effects of Surflan™ and oryzalin are consistent with previously-reported effects of estrogens or xenoestrogens on spermatogenesis, in which necrotic spermatids (Miles-Richardson et al., 1999), inhibition of spermatogenesis (Billard et al., 1981; Jobling et al., 1996; Kinnberg et al., 2001, Sohoni et al., 2001), mild degeneration of testes (Lange et al., 2001), and testicular atrophy (Jobling et al., 1996; Panter et al., 1998) have been reported. These adverse effects on gonad structure and gametogenesis encompass a range of diagnostic criteria and a spectrum of severity of effect. Underlying these differences are the use of separate species, distinct chemicals, different exposure durations, and different dosages. Notwithstanding the existence of these variables, there is considerable consistency in the nature of effects on the male gonad associated with exposure to estrogenic substances. How a xenoestrogen elicits these effects is not clear, but Surflan™ and oryzalin could act directly at the gonad by either suppressing testosterone synthesis, altering testosterones' rate of conversion to estradiol by affecting p450arom, or indirectly via feedback effects at the pituitary that impact the synthesis and secretion of gonadotropins and in turn, affecting steroid levels within the gonad.

The development of ovarian lesions after estrogenic challenge is both more poorly defined and less frequently identified than effects in males. For example, a number of papers have cited an absence of lesions in female fish subsequent to xenoestrogen exposure (Gray et al., 1999b; Yokota et al., 2001; Sohoni et al., 2001). Furthermore, when ovarian effects have been attributed

to estrogenic compounds, no consistent index or set of criteria has been used, making it difficult to compare effects between studies.

The effect of xenoestrogen exposure on ovarian follicular status was evaluated in this study, and was also evaluated by Miles-Richardson et al. (1999). The criteria used to define follicular status were not identical however, in that Miles-Richardson et al. (1999) scored the stage of follicular development for 50 follicles per ovary, and we scored the severity of necrotic follicles (ovarian follicular necrosis, OFN) and the severity of OA (see Table 2-12). We observed no effects of exposure on either OFN or OA, which is generally consistent with the absence of effect on follicular development reported by Miles-Richardson et al. (1999).

The category of IO does not describe a true lesion, but represents a set of criteria that allowed us to evaluate whether exposure affected the early development of oocytes. Both Surflan™ and oryzalin affected the mean lesion severity score for IO, and treatment with oryzalin was associated with a significant decrease in the mean severity score for IO's. The scoring citeria for IO (Table 2-2) were established in a way such that the lower the score, the fewer the number of immature oocytes present in an ovary. Oocytes are derived from mitotically-active oogonia, stem cells that develop from primordial germ cells which migrate into the ovary during embryogenesis. Oogonial proliferation and the increase in oocyte number are followed by development of the oocyte within a follicle. Under the influence of gonadotropins, follicles synthesize testosterone and estradiol; the latter reaction occurs via the p450 arom-mediated conversion of testosterone (Nagahama, 1987). The decrease in the number of immature oocytes in ovaries of exposed females may be a consequence of oryzalin acting to inhibit the formation

of oocytes – either by some direct action on oogonia, or by interference with estrogen or gonadotropin homeostasis. Lange et al. (2001) reported a qualitatively similar effect in ovaries of fathead minnows exposed to 17α-EE2 i.e, a dose-related increase in the number of primary and secondary (immature) oocytes in exposed fish. Similarly, zebrafish exposed to 17α-EE2 had highly reduced ovarian somatic indices and an absence of "yolky oocytes", indicating that 17α-EE2 interferes with oocyte development in this species as well (Van den Belt et al., 2001).

The severity of OMA scores were elevated in four of six treatment groups relative to controls, and neared statistical significance in low-dose Surflan™ females. Evaluation of this lesion by treatment category (treatment with either Surflan™ or oryzalin, all doses combined) did not yield any statistically significant results. The presence of macrophages within the ovaries is common, as macrophages function to remove normal necrotic debris (Hughes and Gorospe, 1991). The apparent treatment-related increase in the severity scores for OMA suggests that treatment increases the number of necrotic cells within an ovary and increases the number of macrophages needed to phagocytize the resulting cellular debris.

Many of the same treatment groups that displayed an apparent decrease in the number of IO's, also exhibited OHH (all oryzalin dose groups as well as the low-dose Surflan™group). We reviewed the lesion score data for individual animals, and in most cases, both IO's and OHH were present in the same ovary. Considering the scores for these two criteria, our data indicates that there was both a decrease in the number of IO's, and an increase in the number of developing oocytes and/or an overall enlargement of the ovary, primarily in oryzalin-treated animals. One possible explanation of this phenomenon is that exposure to oryzalin not only

inhibits the formation of oocytes but slows their rate of development, such that oocytes that are formed remain in the ovary for a longer period, leading to an increase in ovary size. If oocyte development is impaired by treatment with oryzalin, the effect appears to be relatively subtle as oryzalin did not affect the fecundity of treated females (Table 2-5).

Akin to reproduction in males, female teleost reproduction is regulated pituitary hormones that control levels of gonadal steroids critical to ovarian maturation and gametogenesis. In females, estradiol acts to regulate gonadotropin synthesis and secretion (Dickey and Swanson, 1998; Saligaut et al., 1998). Harris et al. (2001) recently demonstrated that female trout exposed to the xenoestrogen nonylphenol (NP) at the onset of seasonal sexual maturation exhibited a markedly lower gonadosomatic index (GSI) than controls. The decrease in GSI was attributed to an apparent complete absence of normal seasonally induced growth and development. In these trout, pituitary mRNA levels of the β subunit of FSH (GtH-I) and mRNA of LH (GtH-II) were significantly lower in treated animals. Plasma FSH was also significantly inhibited by NP. These results are analogous to those described by Blake and Boockfor (1997) in rats exposed to the structurally related compound octylphenol. Thus, it appears that certain xenoestrogens function biologically as estrogen agonists at the level of the pituitary, potentially acting by negative feedback to decrease gonadotropin secretion. Specifically how the gonad of a reproductively active individual fish responds to xenoestrogen-modulated lower GtH levels is not clear, but may involve compensatory responses of p450arom (Scholz and Gutzeit, 2000) and/or 17α-hydroxylase c17/20 lyase (Majdic et al., 1996).

Exposure to Surflan™ or oryzalin affected the fertility of male and female medaka. We were able to demonstrate the statistical significance of these results only in low-dose oryzalin females and in mid-dose Surflan™ and mid-dose oryzalin males. However, exposure to Surflan™ or oryzalin increased the production of nonfertilized eggs (and thus decreased the production of fertilized eggs) in all treatment groups of both genders. The production of nonfertilized eggs was a highly variable event, and was typically manifested as most or all of a given clutch of eggs failing to be fertilized on the day of collection; this tended to alternate with successful fertilization for a period of days, followed by another failed fertilization. This pattern of fertilization failure was observed in all of the treated groups, and yielded fertilization data characterized by substantial variability. This variability in the response of individuals within all treatment groups made it exceptionally difficult to demonstrate statistical significance.

The decreased fertilization rates in treated females observed in this study may be due to a number of factors, including altered egg envelope structure or decreased viability of the oocyte. Both Surflan™ and oryzalin induce the high molecular-weight subunits of the egg envelope proteins, the choriogenins (Cg), (see Section 1), so it is possible that alterations in Cg production occur from exposure and affect the ability of an egg to be fertilized. The treatment-related effects of an apparent decrease in oocyte production (fewer IO's) and delayed oocyte development (increased OHH) may affect either the viability of the oocyte or it's fertilization ability. However, these possible explanations for altered fertility in treated females are entirely speculative at this point. Identification of the underlying cause(s) for reduced fertility will require further study.

Exposure to both Surflan™ and oryzalin was associated with a reduced fertilization capacity of treated male medaka paired with untreated females. This phenomenon has been observed in zebrafish, where an exposure to 17α-EE2 caused a dose-related reduction in the number of unexposed females that spawned when paired with unexposed males (Van den Belt et al., 2001). Male medaka exposed to the xenoestrogen octylphenol (OP) for 6 months were also found to be significantly less likely to fertilize eggs than untreated controls. These latter data, reported within the context of OP-induced effects on behavior, also documented a significant correlation between impaired courtship behavior in treated males and a lower egg fertilization success rate (Gray et al., 1999b). Gronen et al. (1999) also documented significantly lower fertilization rates in male medaka exposed to OP and mated with unexposed females. Fecundity of these breeding pairs was also affected, resulting in the production of approximately 50% fewer eggs than controls. Bjerselius et al. (2001) showed that a relatively brief exposure to estradiol (24-28 d) negatively impacted a number of courtship parameters in treated male goldfish paired with an unexposed, artificially induced spawning female.

Therefore, for males in particular, but also potentially for females, decreased fertility may be due to effects of treatment with estrogenic substances on reproductive behavior. In the brain, p450arom is concentrated in those regions linked to steroid regulation of reproduction and sexual behavior (McEwen et al., 1979). In both goldfish and medaka, brain p450arom activity increased after treatment with exogenous estrogen (Callard et al., 1995; Melo and Ramsdell, 2001), indicating the possibility that brain p450arom is regulated directly by estrogen in both species (Callard et al., 2001). Melo and Ramsdell (2001) have identified a gender-specific pattern of distribution of p450arom in medaka brain. In males exposed to estradiol, brain

p450arom activity increased, and the distribution pattern shifted such that it resembled the female phenotype. The implication of these data is that changes in p450arom activity and distribution may be causally linked with altered reproductive behavior and fertility. These explicit correlations have not been made, experimentally, however.

Total daily mean egg production was not affected by exposure to Surflan™ or oryzalin. There are reports that document an absence of an effect on fecundity in fish exposed to a xenoestrogen or to the pharmaceutical estrogen, 17α-EE2 (Yokota et al., 2001; Lange et al., 2001). There are other studies however, that indicate that exposure to estrogens or a xenoestrogen adversely affects egg production. Kramer et al. (1998) described a concentration-related decrease in egg production of fathead minnows exposed as breeding pairs to 17β-estradiol. In zebrafish, 17α-EE2 caused a dose-related decrease in the number of spawning females and in the number of unexposed females that spawned when paired with exposed males (Van den Belt et al., 2001).

There were statistically significant differences in the time-to-hatch of eggs from all groups of males and females treated with Surflan™ and oryzalin (Table 2-7). In all Surflan™ and oryzalin dose groups, eggs produced by untreated females paired with treated males took significantly longer to hatch than eggs from controls, and eggs produced by treated females paired with untreated males hatched significantly earlier than controls. It is difficult to speculate on the bases for the observed alterations in time-to-hatch, especially considering that egg hatching was affected differently depending on the gender of the exposed parent. Nonetheless, for exposed medaka of both genders, the effects on time-to-hatch were remarkably consistent, occurring in all dose groups. The biological significance of these effects is not clear, given that survival of

progeny were not affected by exposure (Table 2-8), nor were there any effects on the incidence of deformities in juveniles grown to approximately 30 d post-hatch. Metcalfe et al. (2000) found that eggs of female medaka exposed to the xenoestrogen o,p′-DDT and paired with unexposed males exhibited a significant delay in hatching time. As in this study, the impact on hatching time was not associated with any effects on mortality.

Given the many aspects of reproduction regulated by estrogen and thus subject to perturbation by xenoestrogens, multiple factors may be involved in effects seen in a specific measurement endpoint such as fertilization success. Although we documented gonadal lesions in both males and females and altered fertilization success in both genders, there is not a complete correspondence between the groups that manifested the most severe lesions and the groups that exhibited the greatest effects on fertility. However, in general, treatment with oryzalin at 0.5 mg/l or 0.25 mg/l had the greatest adverse effects on male and female fertility as well as on the severity of non-intersex gonadal lesions.

The maximum dose of oryzalin and maximum concentration of Surflan™ used to assess reproductive toxicity were selected on the basis of range-finding studies that identified an apparent MTD for each compound. Mid- and low-dose amounts of Surflan™ and oryzalin were set at approximately 50% decrements of the MTD or of the mid-dose quantity, respectively. Surflan™ is an emulsion that contains 40.4% oryzalin, and at the concentrations used, gave nominal doses of oryzalin of 1.5 mg/l, 0.8 mg/l, and 0.4 mg/l, nominal concentrations that are approximately 60% higher than were administered for oryzalin given alone. Despite the administration of greater amounts of oryzalin in the Surflan™ groups, in general, Surflan™ did

not elicit more severe effects than the lower doses of oryzalin administered alone. The one clear exception to this is seen in the prevalence and severity data for induction of intersex lesions, where Surflan™ consistently caused more severe lesions as well as a greater prevalence of these lesions than oryzalin alone. It is also noteworthy that the high-dose oryzalin group (1 mg/l) had no statistically significant or marginally significant effects on non-intersex testicular lesions or on any measure of male or female fertility, and in only one category of ovarian lesion, OHH, did effects approach statistical significance. In fact, the most severe lesions often occurred in the low-dose oryzalin group, or the effects in that group were similar in magnitude to those observed from treatment with the next higher dose. Similarly, the highest concentration of Surflan™ was not associated with significant effects on non-intersex testicular lesions or on any ovarian lesion. There were marginally significant effects of high-dose Surflan™ on total egg production in treated females or in F+M considered together. These results suggest that oryzalin administered alone, or administered in Surflan™, has a distinctly non-linear dose response for the reproductive parameters we assessed. This phenomenon has been documented for certain other xenoestrogens, but is not a universal characteristic of these compounds (Andersen et al, 1999; Melnick et al., 2002). The National Toxicology Program's Panel on low-dose effects and nonmonotonic dose-response relationships for endocrine-disrupting chemicals (EDCs) focused on mammalian testing of EDCs, and did not extend their review to fish. Nonetheless, the panel concluded that current testing 'paradigms' for the assessment of reproductive and developmental toxicity may require reassessment to account for this fact (Melnick et al., 2002). It is clear from our data that we have not defined a lowest-observed-adverse- effect-level for reproductive effects of oryzalin and Surflan™; further testing to evaluate the consequences of exposure to

environmental levels of oryzalin must consider the dose-response phenomenon suggested by our data and structure studies accordingly.

Conclusion

A relatively brief (21 d) exposure of mature, reproductively-active medaka to Surflan™ and oryzalin impaired fertilization of both males and females, and affected the hatching time of eggs. Fecundity, viability of eggs, and incidence of deformities of offspring were not affected by exposure.

Exposure to Surflan™ or oryzalin clearly increased the prevalence and severity of intersex lesions, increased the severity of NST and NSG (and NST and NSG combined), although only those effects on NST and NSG associated with oryzalin exposure were statistically significant. Whether the testicular lesions caused by exposure led directly to the observed decrements in fertility in males, or whether there are indirect effects of treatment that adversely affect fertility – such as impairment of reproductive behavior–are not known.

Oryzalin affected the number of immature oocytes present in the ovaries of exposed females. We suspect that this effect may be due to effects on oogonia, such that oryzalin may be inhibiting the ability of oogonia to mitotically divide to form oocytes. Whether this specific hypothesis is correct or not, oryzalin does appear to inhibit the formation of oocytes, resulting in fewer numbers of IO's in the ovaries of exposed females. Oryzalin also adversely affected spermatogonia, and thus it appears that oryzalin may be capable of affecting the function of germ cells of both males and females. Although the mechanism(s) of these effects remains to be

identified, it appears plausible that a phenomenon experienced by both genders – such as alteration in estrogen homeostatis– may underlie the toxicity elicited by these compounds.

Table 2-1. Testicular Lesions and Lesion Scoring Criteria for Male Medaka.

Testicular Lesion	**Lesion Severity Score (0-3) and Score Criteria**[a]			
	0	**1**	**2**	**3**
Pre-intersex lesion (PIL)	no lesion present	< 2 PIL cells	2–5 PIL cells	> 5 PIL cells
Intersex Testes, Stage 1 Oocytes (S1-Oo)	**0** no oocytes present in the testes	**1** < 1 S1-Oo	**2** 1-2 S1-Oo clusters[b]	**3** > 2 S1-Oo clusters[b]
Intersex Testes, Stage 2/3 Oocytes	**0** no oocytes present in the testes	**1** 1 or 2 stage 3 oocytes per testes	**2** ≥3, stage 3 single oocytes or one small oocyte cluster[b] per testes	**3** 2 or more clusters[b] of stage 3 oocytes per testes
Testicular Sperm Depletion	**0** no sperm depletion; seminiferous tubules in the medullary zone; the ductus deferens are large, dilated, and packed with mature spermatozoa	**1** mild sperm depletion; seminiferous tubules and ductus deferens have 70-90% of normal[c] sperm production	**2** moderate sperm depletion; tubules and ductus deferens have 30-60% of normal[c] sperm production	**3** severe sperm depletion; tubules and ductus deferens have <30% of normal[c] sperm production; tubules are only partially filled, small, and have a patchy appearance

Table 2-1. Testicular Lesions and Lesion Scoring Criteria for Male Medaka, con't.

Testicular Lesion	Lesion Severity Score (0-3) and Score Criteria[a]			
Necrosis, spermatids	**0**	**1**	**2**	**3**
	no necrotic spermatids	mild spermatid necrosis; <2 dead spermatids per 40x field	moderate spermatid necrosis; 2-20 dead spermatids per 40x field	severe spermatid necrosis; >20 dead spermatids per 40x field
Necrosis, spermatids	**0**	**1**	**2**	**3**
	no necrotic spermatogonia	mild necrosis of spermatogonia; 2-10 dead spermatogonia per 40x field	moderate necrosis of spermatogonia; 2-20 dead spermatogonia per 40x field	severe necrosis of spermatogonia; >10 dead spermatogonia per 40x field

[a] Score criteria refer to the number of cells or lesions per half a 40 x microscope field.
[b] Oocyte cluster is defined as 5 or more oocytes.
[c] Normal was defined on the basis of levels of sperm production in two type specimens.

Table 2-2. Ovarian Lesions and Lesion Scoring Criteria for Female Medaka.

Ovarian Lesion	**Lesion Severity Score (0-3) and Score Criteria**			
	0	**1**	**2**	**3**
Ovarian Macrophage Aggregates (OMA)	no macrophage aggregates	< 3 macrophage aggregates per 20x field	3 –10 macrophage aggregates per 20x field	> 10 macrophage aggregates per 20x field
Ovarian Follicular Necrosis	**0** no necrotic follicles	**1** < 4 necrotic follicles per four 10x fields	**2** 4-6 necrotic follicles per four 10x fields	**3** > 6 necrotic follicles per four 10x fields
	0	**1**	**2**	**3**
Oocyte Atresia	no atretic oocytes present	< 3 atretic oocytes per ovary	3-5 atretic oocytes per ovary	> 5 atretic oocytes per ovary
Ovarian Hypertrophy /Hyperplasia	**0** ovary < 20% of abdominal cavity	**1** ovary 20-30% of abdominal cavity	**2** ovary 31-40% of abdominal cavity	**3** ovary > 40% of abdominal cavity

Table 2-2. Ovarian Lesions and Lesion Scoring Criteria for Female Medaka, cont.

Ovarian Lesion	Lesion Severity Score (0-3) and Score Criteria			
Immature Oocytes	**0**	**1**	**2**	**3**
	immature oocytes < 5% of total ovarian oocytes	immature oocytes 5-10 % of total ovarian oocytes	immature oocytes 11-50 % of total ovarian oocytes	immature oocytes > 50 % of total ovarian oocytes

Table 2-3. Daily mean production of non-fertilized eggs in medaka after exposure to either Surflan™ or oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure concentration	Treated medaka Females and Males [a]	Treated Females [b]	Treated Males [c]
Control	1.7	1.7	1.8
Surflan™ 2.5 µl/l	3.7 n.s.	3.1 n.s.	4.4 n.s.
Surflan™ 1.3 µl/l	3.7 **	2.9 n.s.	4.6 **
Surflan™ 0.67 µl/l	2.7 n.s.	2.3 n.s.	3.2 n.s.
Oryzalin 1 mg/l	5.1 n.s.	5.3 n.s.	4.9 n.s.
Oryzalin 0.5 mg/l	4.8 ***	4.5 *.	4.9 ***
Oryzalin 0.25 mg/l	4.7 **	5.2 *	4.2 n.s.

[a] Data are from 12 replicates; 6 containing treated males (paired with untreated females) or 6 treated females (paired with untreated males). Each replicate contained 2 males and 2 females. Kruskall-Wallace ANOVA results for Treated medaka, Females and Males: F ratio, 1.646; p = 0.133; DF = 6

[b] Data are from 6 replicates containing treated females paired with untreated males. Each replicate contained 2 males and 2 females. Kruskall-Wallace ANOVA results for Treated Females: F ratio, 1.102; p = 0.362; DF = 6

[c] Data are from 6 replicates containing treated males paired with untreated females. Each replicate contained 2 males and 2 females. Kruskall-Wallace ANOVA results for Treated Males: F ratio, 0.748; p = 0.612; DF = 6

n.s. = not significant.

* $p < 0.05$. Not significant by the criterion for statistical significance ($p < 0.008$) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.

** $p < 0.005$

*** $p < 0.001$

Table 2-4. Mean daily total egg production of medaka after exposure to either Surflan™ or oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure concentration	Treated medaka Females and Males [a]	Treated Females [b]	Treated Males [c]
Control	28.0	30.8	25.1
Surflan™ 2.5 µl/l	23.3 *	23.6 *	23.0 n.s.
Surflan™ 1.3 µl/l	26.1 n.s.	24.8 n.s.	27.4 n.s.
Surflan™ 0.67 µl/l	28.8 n.s.	29.7 n.s.	27.8 n.s.
Oryzalin 1 mg/l	28.4 n.s.	35.6 n.s.	21.1 n.s.
Oryzalin 0.5 mg/l	28.2 n.s.	31.7 n.s.	24.7 n.s.
Oryzalin 0.25 mg/l	22.8 *	26.9 n.s.	18.6 *

[a] Data are from 12 replicates; 6 containing treated males (paired with untreated females) or 6 treated females (paired with untreated males). Each replicate contained 2 males and 2 females. Kruskall-Wallace ANOVA results for Treated medaka, Females and Males: F ratio, 1.81; p = 0.095; DF = 6

[b] Data are from 6 replicates containing treated females paired with untreated males. Each replicate contained 2 males and 2 females. Kruskall-Wallace ANOVA results for Treated Females: F ratio, 2.44; p = 0.0269; DF = 6

[c] Data are from 6 replicates containing treated males paired with untreated females. Each replicate contained 2 males and 2 females. Kruskall-Wallace ANOVA results for Treated Males: F ratio, 1.72; p = 0.1164; DF = 6

n.s = not significant.

* $p < 0.05$. Not significant by the criterion for statistical significance ($p < 0.008$) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.

Table 2-5. Mean percentage of eggs hatched, compared by treatment group.

Exposure concentration	Treated medaka		
	Females and Males [a]	Treated Females [b]	Treated Males [c]
Control	0.85	0.89	0.82
Surflan™ 2.5 µl/l	0.95 n.s.	0.96 n.s.	0.95 n.s.
Surflan™ 1.3 µl/l	0.91 n.s.	0.88 n.s.	0.94 n.s.
Surflan™ 0.67 µl/l	0.93 n.s.	0.97 n.s.	0.90 n.s.
Oryzalin 1 mg/l	0.84 n.s.	0.94 n.s.	0.74 n.s.
Oryzalin 0.5 mg/l	0.93 n.s.	0.91 n.s.	0.95 n.s.
Oryzalin 0.25 mg/l	0.91 n.s.	0.93 n.s.	0.89 n.s.

[a] Data are from 12 replicates; eggs were collected from 6 replicates containing treated males (paired with untreated females) or from 6 replicates containing treated females (paired with untreated males). Each replicate contained 2 males and 2 females.
[b] Data are from 6 replicates containing treated females paired with untreated males. Each replicate contained 2 males and 2 females.
[c] Data are from 6 replicates containing treated males paired with untreated females. Each replicate contained 2 males and 2 females.
n.s. = not significant.

Table 2-6. Results of time-to-hatch data evaluated by the Wald-Wolfowitz Test. All results are statistically significant ($p < 0.05$).

Exposure concentration	Treated Females [a]	Treated Males [b]
	No. of runs (sign of outcome)	
Surflan™ 2.5 µl/l	2 (+)	1 (–)
Surflan™ 1.3 µl/l	2 (+)	3 (–)
Surflan™ 0.67 µl/l	2 (+)	3 (–)
Oryzalin 1 mg/l	2 (+)	3 (–)
Oryzalin 0.5 mg/l	4 (+)	1 (–)
Oryzalin 0.25 mg/l	4 (+)	2 (–)

[a] Data are from 6 replicates containing treated females paired with untreated males. Each replicate contained 2 males and 2 females.
[b] Data are from 6 replicates containing treated males paired with untreated females. Each replicate contained 2 males and 2 females.

Table 2-7. Mean testicular lesion severity scores in medaka exposed to Surflan™ or oryzalin. Lesions and lesion scoring criteria are defined in Table 2-1. Data were analyzed by one-way ANOVA to determine statistical significance between mean scores of treated and control groups.

	Intersex lesions						
Treatment group [a]	Pre-intersex lesion	Stage 1 oocyte	Stage 2/3 oocyte	Stages 1 & 2/3 oocytes	NST[b]	NSG[c]	TSD[d]
Control	0.33	0.17	0.00	0.2	1.00	0.83	1.33
Surflan™ 2.5 µl/l	0.67 n.s.	1.50*	0.67*	2.2*	1.17 n.s.	1.33 n.s.	1.33 n.s.
Surflan™ 1.3 µl/l	0.40 n.s.	0.60 n.s.	0.20 n.s.	0.8*	2.00 n.s.	1.80*	2.20 n.s.
Surflan™ 0.67 µl/l	0.67 n.s.	1.33**	0.33 n.s.	1.7*	1.33 n.s.	0.83 n.s.	1.83 n.s.
Oryzalin 1 mg/l	0.67 n.s.	0.83 n.s.	0.17 n.s.	1.0 n.s.	1.33 n.s.	1.33 n.s.	1.50 n.s.
Oryzalin 0.5 mg/l	0.83 n.s.	0.67 n.s.	0.83 n.s.	1.5*	2.17*	2.0*	1.33 n.s.
Oryzalin 0.25 mg/l	0.50 n.s.	0.33 n.s.	0.33 n.s.	0.7 n.s.	2.17 n.s.	2.0**	0.83 n.s.

[a] Scores are the mean of observations on 6 males per treatment group.
[b] NST refers to necrosis of spermatids
[c] NSG refers to necrosis of spermatogonia
[d] TSD refers to testicular sperm depletion.
* $p < 0.05$
** $p < 0.005$
n.s. not significant

Table 2-8. Mean testicular lesion scores in medaka compared by category of treatment.

Treatment category	PIL, S1-Oo, and S2/3 Oo [a]	S1-Oo and S2/3 Oo [a]	NST [b]	NSG [c]	NST & NSG [b, c]
Control [d]	0.5	0.2	1.0	0.83	1.83
Oryzalin [e]	1.72	1.05	1.89 [f]	1.78 [f]	3.67 [f]
Surflan™ [e]	2.18 [f]	1.59 [f]	1.47	1.29	2.76

[a] PIL, S1-Oo, and S2/3 Oo refer to Pre-intersex lesions (PIL), testes with stage 1 oocytes (S1-Oo), or testes with stage 2/3 oocytes (S2/3 Oo).
[b] NST refers to necrotic spermatids.
[b] NSG refers to necrotic spermatogonia.
[d] Scores are the mean of observations on 6 males per treatment group.
[e] Scores are the mean of observations on 18 males per treatment group.
[f] Result is statistically significant ($p < 0.05$) by one way ANOVA.

Table 2-9. Mean ovarian lesion scores in medaka exposed to Surflan™ or oryzalin.

Treatment group[a]	Ovarian macrophage aggregates	Oocyte atresia	Ovarian follicular necrosis	Immature oocytes	Ovarian hyperplasia or hypertrophy
Control	1.83	0.17	1.83	1.50	1.83
Surflan™ 2.5 µl/l	2.33 n.s.	0.50 n.s.	1.33 n.s.	1.17 n.s.	2.17 n.s.
Surflan™ 1.3 µl/l	1.83 n.s.	0.00 n.s.	1.83 n.s.	1.50 n.s.	2.17 n.s.
Surflan™ 0.67 µl/l	2.67*	0.00 n.s.	1.83 n.s.	1.17 n.s.	2.67*
Oryzalin 1 mg/l	1.83 n.s.	0.33 n.s.	2.50 n.s.	0.67 n.s.	2.67*
Oryzalin 0.5 mg/l	2.17 n.s.	0.17 n.s.	1.83 n.s.	0.67 n.s.	3.00*
Oryzalin 0.25 mg/l	2.17 n.s.	0.17 n.s.	1.83 n.s.	0.67 n.s.	2.67*

[a] Scores are the mean of observations on 6 females per treatment group.
* $p < 0.05$. Not significant by the criterion for statistical significance ($p < 0.008$) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.
n.s. not significant.

Table 2-10. Mean ovarian lesion scores in medaka compared by category of treatment.

Treatment category	Ovarian macrophage aggregates	Oocyte atresia	Ovarian follicular necrosis	Immature oocytes	Ovarian hyperplasia or hypertrophy
Control [a]	1.83	0.17	1.83	1.50	1.50
Oryzalin [b]	2.10 [n.s.]	0.17 [n.s.]	2.01 [n.s.]	0.67[c]	2.80 [d]
Surflan™ [b]	2.30 [n.s.]	0.17 [n.s.]	1.67 [n.s.]	1.28 [n.s.]	2.30 [n.s.]

[a] Scores are the mean of observations on 6 females per treatment group.
[b] Scores are the mean of observations on 18 females per treatment group.
[c] Result is statistically significant (p = 0.01) by one way ANOVA.
[d] Result is statistically significant (p = 0.007) by one way ANOVA.

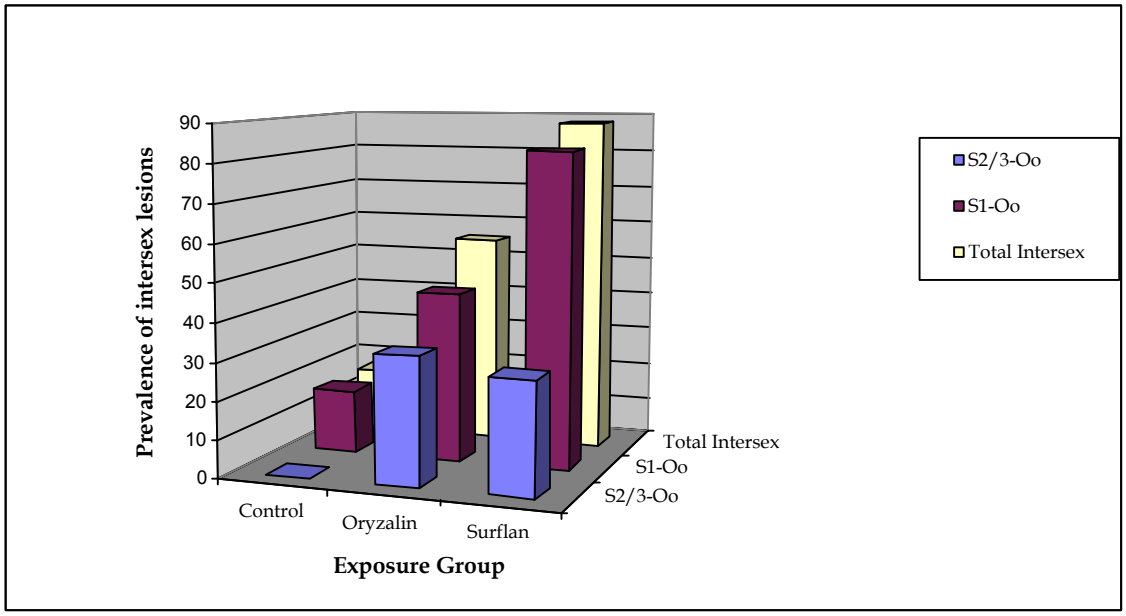

Figure 2-1. Prevalence of intersex lesions in testes of control medaka and those exposed to oryzalin and Surflan™. S1-Oo = stage 1 oocytes; S2/3-Oo = stage 2 or 3 oocytes; Total Intersex = combined stage 1 and stage 2 or 3 intersex lesion severity scores

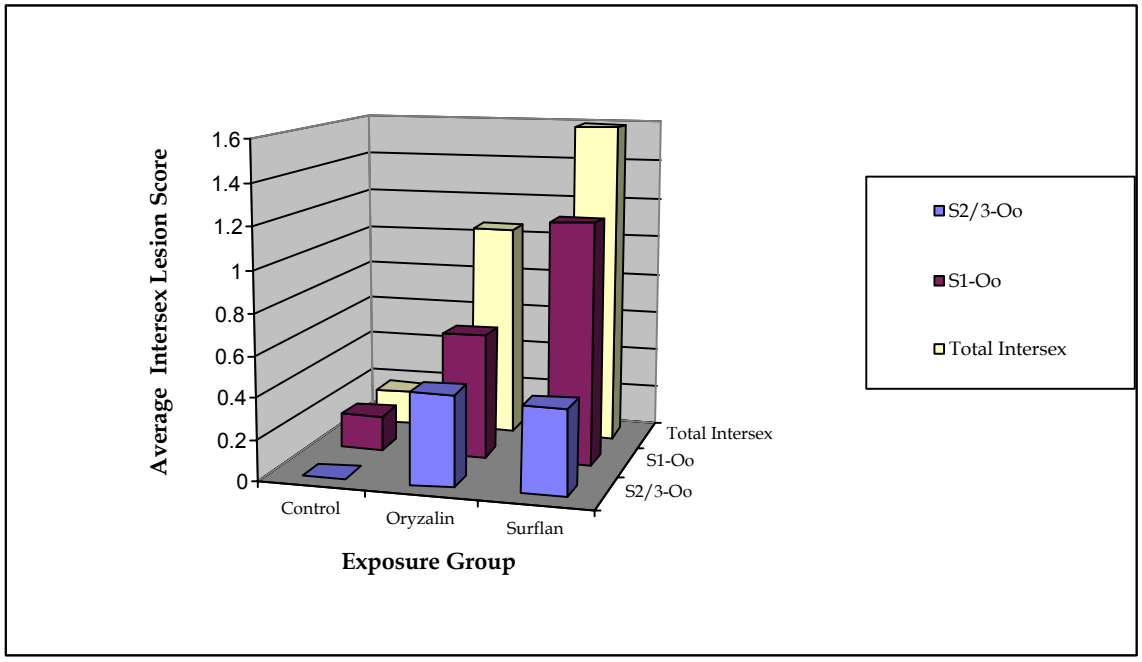

Figure 2-2. Average intersex lesion severity scores for testes of control medaka and those exposed to oryzalin and Surflan™. S1-Oo = stage 1 oocytes; S2/3-Oo = stage 2 or 3 oocytes; Total Intersex = combined stage 1 and stage 2 or 3 intersex lesion severity scores.

SECTION III

MECHANISTIC INDICATORS OF TOXICITY OF ORYZALIN

In Section 1, the herbicide Surflan™ and its active ingredient oryzalin (3,5-dinitro-N4, N4-dipropylsulfanilamide) were characterized as xenoestrogens. Oryzalin competitively displaced 17-β estradiol from the estrogen receptor (ER), both Surflan™ and oryzalin interacted with estrogen response elements (ERE) *in vitro*, and induced expression of the estrogen-regulated genes encoding high molecular weight choriogenins (egg envelope proteins) in medaka (*Oryzias latipes*). In Section 2, we demonstrated that both Surflan™ and oryzalin are reproductive toxicants that adversely affect fertilization success in male and female medaka and induce gonadal lesions in males. The ability of Surflan™ and oryzalin to affect reproduction in both genders indicates the possibility that toxicity may occur through biochemical and/or molecular mechanisms common to both sexes. As chemicals with estrogenic activity, perturbation of estrogen homeostasis by Surflan™ and oryzalin appears to be a likely cause for the observed adverse reproductive effects; we hypothesized that Surflan™ and oryzalin may affect estrogen synthesis and ER concentration in tissues critical to reproduction. Modulation of estrogen biosynthesis and ER expression are documented targets of xenoestrogens (Flouriot et al., 1995; Yadetie et al., 1999; Scholz and Gutzeit, 2000; Kishida et al., 2001; Melo and Ramsdell, 2001).

In teleosts, estrogen regulates vitellogenesis and choriogenin synthesis (Chester-Jones et al., 1987, Murata et al., 1994, 1997) in females; sexual maturation (Yamamoto, 1969; Goetz et al., 1979; Van den Hurk et al., 1982), and reproductive behavior (Gray et al. 1999, Bjerselius et al. 2001) in both sexes. In all vertebrates, estrogen plays a number of critical roles in sexual differentiation of the brain (Chester-Jones et al. 1987; Beyer, 1999). Estrogen is synthesized

locally in the ovaries, testes, and brain of teleosts by the conversion of androgens to estrogen (Chester-Jones et al. 1987; Pasmanik and Callard, 1988). The rate-limiting step in estrogen synthesis is the cytochrome p450 aromatase (p450arom)-mediated conversion of the A-ring of C-19 steroid precursors (e.g., testosterone) to C-18 estrogens (Simpson et al., 1994; Conley and Hinshelwood, 2001). Conversion of testosterone to estradiol by p450arom is critical to the biological activity of testosterone in the central nervous system (McEwen et al. 1979; Callard et al., 1987; 1990), and brain p450arom appears to be localized to those regions of the brain involved in reproduction and sexual behavior (McEwen et al. 1979; Parsons et al., 1984; Pasmanik and Callard, 1988). In mammals, p450arom is expressed in testicular Sertoli and Leydig cells (Valladares and Payne, 1979; Janulis et al., 1996). p450arom is also present in spermatogonia and epididymal sperm; spermatogenesis was disrupted in mice lacking a functional aromatase gene, resulting in the arrest of early spermiogenesis and ultimately impairing fertility (Robertson et al., 1999). Limited data have demonstrated the presence of mammalian hepatic p450arom (Biegel et al., 1995; Liu et al., 1996; You et al., 2001); with the exception of the present study, hepatic p450arom has not been reported in teleosts (Gelinas et al., 1998; Kwon et al., 2001). Studies with rainbow trout (Tanaka et al., 1992), medaka (Fukada et al., 1996), tilapia (Chang et al., 1997) and red seabream (Gen et al., 2001) have shown that mRNA levels of p450arom are increased in association with aromatase enzyme activity during vitellogenesis and oocyte development.

Regulation of p450arom expression is highly complex. Human p450arom exists as a single isoform, with tissue-specific promoters that regulate differential responsiveness to biochemical regulators of transcription (Means et al., 1991; Simpson et al., 1993). p450arom activity is

correlated with changes in the levels of mRNA encoding the p450arom gene (Means et al., 1991). In mammals, induction of gonadal p450arom expression by pituitary gonadotropins (GtHs) is regulated by promoters that confer responsiveness to cAMP. In ovary and testis, the GtHs act through increasing the concentration of cAMP in granulosa and Leydig cells to induce expression of p450arom (Valladares and Payne, 1979; Simpson et al., 1994; Conley and Hinshelwood, 2001). Regulation of mammalian brain p450arom differs from that in gonads, in that p450arom expression in brain is increased by aromatizable androgens, and is either suppressed or not affected by cAMP (Lephart et al., 1996; Simpson et al., 1997). In the teleosts goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*), two isoforms of p450arom exist; these isoforms are differentially expressed in the ovary (cyp19a); and brain, pituitary, and retina (cyp19b) (Gelinas et al., 1998; Callard et al., 2001). The identification of two EREs on the cyp19b gene suggests that transcription of this p450arom isoform is regulated directly by estrogen (Callard et al., 2001). Goldfish and medaka ovarian p450arom genes lack complete EREs, but contain a steroidogenic factor (SF-1 or Ad4BP) binding site (Tanaka et al., 1995; Callard et al., 2001). The SF-1 sequence confers cAMP–responsiveness to the gene (Tanaka et al., 1995; Simpson et al., 1997; Callard et al., 2001). cAMP–responsiveness of teleost ovarian p450arom is consistent with the induction of p450arom *in vitro* by incubation of early-vitellogenic follicles with GtH or drugs that increase intracellular levels of cAMP (Nagahama, 1987; Nagahama et al., 1991). To date, ovarian p450arom is the only aromatase gene characterized in medaka (Tanaka et al., 1995). Whether or not tissue-specific isoforms of p450arom exist in medaka remains to be determined. It is intriguing to note however, that both goldfish and medaka brain p450arom activity increased after treatment with exogenous estrogen

(Callard et al., 1995; Melo and Ramsdell, 2001), indicating the possibility that brain p450arom is regulated directly by estrogen in both species (Callard et al., 2001).

Historically, estrogens have been considered primarily female reproductive hormones, with ample data demonstrating their synthesis in ovarian tissue and their critical roles in oogenesis (Chester-Jones et al., 1987). Considerable data exists which indicate estrogen has a critical role in male reproduction as well. In mammals, estrogen regulates testicular androgen production by controlling androgen synthesis with the formation of 17-α hydroxylase, C17/20 lyase complex (Dufau, 1988). Additionally, ERs have been detected in testes and sperm of humans, rodents, and other vertebrate species (Kato et al., 1974, Murphy et al., 1980, Mak et al., 1983 a, b; Callard and Mak 1985; Cooke et al., 1991). In rodents and humans, those ERs are of two forms; ER-α and ER-β. Directed mutation of ER-α yielded male mice that were infertile, with sperm of young adults unable to fertilize eggs *in vitro* (Mahato et al. 2001); ER-β does not appear to have a critical role in mammalian male reproduction (Couse et al., 2001). The number, identity, and specific function of the ER(s) in medaka remain uncertain, with only a single ER identified to date (Kawahara and Yamashita 1999).

The ER, present in target tissues of pituitary, brain, liver, and reproductive tract, is a transcription factor activated by binding of ligand i.e., estrogens or estrogen-agonists; the receptor-ligand complex subsequently interacts with an ERE proximal to an estrogen-regulated gene (Klinge, 2001) and controls the rate of gene transcription (Pakdel et al. 1991). Estrogen regulates transcription of its own receptor, and estrogen-responsive tissues are regulated both by alterations in receptor number and in plasma estrogen concentration (Scott and Sumpter 1989, Campbell et al. 1994, Le Drean et al., 1995).

Treatment of rainbow trout or juvenile salmon *in vivo*, or incubation of trout primary hepatocyte cultures with estradiol induced a two- to eight-fold increase in hepatic ER mRNA (Mommsen and Lazier, 1988; Pakdel et al., 1991; Salbert et al., 1993; Yadetie et al., 1999). Similar results have been achieved with the xenoestrogens nonylphenol (NP) (Flouriot et al., 1995; Yadetie et al., 1999) and chlordecone, Aroclor 1245, and lindane (Flouriot et al., 1995). Nearly eight-fold induction of ER mRNA has been reported in livers of the teleosts sculpin, sea raven, winter flounder, and Atlantic salmon injected with estradiol (MacKay et al., 1996). Estradiol also induced a two-fold increase in hypothalamic ER mRNA in rainbow trout (Salbert et al., 1993). This upregulation of ER mRNA by exogenous estradiol appears to be characteristic of fish (Counis et al., 1987; MacKay et al., 1996), and is in marked contrast to the downregulation of ER by estradiol in mammals (Counis et al., 1987).

Induction of ER mRNA and ER binding activity by estrogen may be characteristic of oviparous vertebrates (Hayward et al., 1980; Barton and Shapiro, 1988). Administration of exogenous estrogen to rainbow trout or cultured trout hepatocytes increased expression of ER mRNA and enhanced estradiol binding to the ER (Pakdel et al., 1991; Flouriot et al., 1993; Mackay and Lazier, 1993). However, the duration of estradiol-induced effects on ER expression is well-characterized. In rainbow trout, hepatic ER and ER mRNA were induced in parallel within a few hours of a single dose of estradiol, and recovered to basal levels approximately two weeks later (Pakdel et al., 1991). Although the effect of estradiol on trout ER may be transient in the absence of continued stimulus, in *Xenopus*, ER induction in liver appears to be permanent after estradiol exposure (Barton and Shapiro, 1988).

Estrogen is a critical regulator of reproduction in male and female vertebrates. The biological actions of estrogen, mediated by the ER, indicate that changes in ER expression have the potential to alter the transcription of estrogen-sensitive genes. Likewise, p450arom – as the primary source of endogenous estrogens – is clearly a key component in the control of estrogen-regulated gene transcription. Thus, alterations in expression of the ER and p450arom have the potential to disrupt normal reproductive function. Our reproductive study (see Section 2), in which Surflan™ and oryzalin induced adverse effects on fertilization success, are consistent with previously documented effects of estrogen or estrogen agonists on reproduction (Billard et al. 1981; Dufau, 1988; Yasuda et al. 1985; Guillette et al. 1994; MacLatchy and Van Der Kraak 1995; Jobling et al. 1996; Toppari et al. 1996; Nimrod and Benson, 1998; Gronen et al., 1999; Gray et al. 1999a). Changes in expression of the ER and p450arom have considerable explanatory potential in understanding the adverse effects of Surflan™ and oryzalin on medaka reproduction.

Real-time TaqMan™ (Applied Biosystems, Foster City, CA) quantitative polymerase chain reaction (PCR) is a highly sensitive technique that allows the specific enzymatic amplification of cDNA (Leutenegger, 2001; Walker, 2001). With the TaqMan™ system, real-time detection of PCR product can be directly related to the original quantity of sample mRNA (Leutenegger, 2001). The purpose of this study is to use TaqMan™ PCR to quantify expression of medaka ER mRNA and p450arom mRNA. The specific objectives are (1) to characterize and quantify basal expression and chemical-induced alterations in expression of ER mRNA and p450arom mRNA in the brain, gonad, and liver of male and female medaka; and (2) to determine if ER mRNA and

p450arom mRNA expression, measured after an exposure period of 24h, 48h, and 21d, could be correlated with adverse reproductive effects induced by 21-d exposures to Surflan™ and oryzalin (Section 2).

Materials and Methods

Animals

The medaka used in this study were the golden strain of *Oryzias latipes*, originally purchased from Carolina Biological Supply. The colony was established at the University of California, Davis (UCD) in 1986, and has been maintained at the UCD Aquatic Center since that time. The colony's brood stock has been out-crossed twice since 1986 with wild-type Northern strain golden medaka to prevent inbreeding (Davis, 2001).

Fish maintenance and exposure

Five-month-old medaka were separated into seven treatment groups with six replicates per group. Each replicate consisted of two male and two female medaka in a one-L aerated beaker. Fish were maintained in U.S. EPA reconstituted water (Horning and Weber, 1985) at pH 7.2 for a 2-d acclimation period prior to initiation of exposure. During acclimation and exposure, medaka were maintained at 25 $\pm$ 1°C under a photoperiod of 16h light:8h dark, and were fed live brine shrimp at approximately 3.5% of their bodyweight per day. Light was provided at 400-450 lux by Vita-Lite (Coralife Chromatic™) bulbs. Medaka were exposed to nominal concentrations of Surflan™ (DowElanco) (2.5, 1.25, or 0.62 µl/l) or oryzalin (1.0, 0.5, or 0.25 mg/l). Exposure concentrations for oryzalin were identical to those used in the reproductive toxicity study (Section 2). Exposure concentrations of Surflan™ were selected to yield concentrations of oryzalin equivalent to those received by animals dosed with the pure compound. Solutions were prepared fresh daily, with the solution in each beaker completely replaced each day. At the end

of the 24h exposure period, one female and one male medaka were sacrificed from each of three replicates. The remaining male and female from those replicates were sacrificed at 48h. Medaka in the remaining replicates (three replicates per treatment group) were sacrificed after 21d. Medaka were euthanized by immersion in tricaine methane sulfonate (MS222, Sigma). Whole brains, livers, and gonads were dissected, and frozen individually in liquid nitrogen. Tissues were stored at –80°C for approximately two weeks prior to RNA isolation.

RNA isolation and cDNA preparation

Total RNA was isolated using a Qiagen RNeasy™ Mini Kit or Qiagen RNeasy™ 96 Kit. For RNA isolation performed using a RNeasy™ Mini Kit, frozen whole tissues were individually pulverized on dry ice using a microtube pestle (USA Scientific). 350µl of RLT buffer (Qiagen) was added to each sample vial, and the tissue resuspended by pipetting until a homogenous solution was achieved. For RNA isolations performed using a RNeasy™ 96 Kit, a 96-well Master Block Plate (Qiagen) was loaded with two stainless steel beads per well and placed on dry ice for approximately 30 min to cool the plate and minimize RNA degradation. Frozen individual tissues were then loaded into each well, and 300µl of RLT buffer (Qiagen) added to each well. The plate was kept on dry ice during tissue loading and addition of the buffer. Tissues were homogenized mechanically (500 strokes/min for 2 min.) in a Spex CertiPrep 2000 GenoGrinder. Once the tissues were homogenized in RLT buffer, RNA was isolated according to the manufacturers protocols with minor modification where RNA was eluted directly into a collection tube containing 1 µl of RNase-free DNase (Amersham Pharmacia) to remove contaminating DNA. cDNA was prepared by adding a 10µl aliquot of a mastermix containing Random Primers, Superscript^{TM} II RNase H^{-}Reverse Transcriptase with 5x First Strand Buffer,

0.1M DTT, and 10µM RNaseOUT™ (all from Invitrogen); a solution of dNTP's (prepared from 100µM solutions of dTTP, dCTP, dATP, and dGTP; GibcoBRL), and nuclease-free water (Sigma) to 10µl of RNA. The mixture was reacted at 42°C for 50 min, then 80µl of nuclease-free water was added to the sample, and the sample vortexed. Samples were incubated at 95°C for 5 min., cooled on ice, centrifuged briefly to collect the contents, and stored at –20°C.

Quantitative Polymerase Chain Reaction (PCR)

Real-time TaqMan™ PCR systems were optimized to quantify transcripts for the medaka target genes cytochrome p450arom (GenBank Accession No. D82968) and the estrogen receptor (GenBank Accession No. 6451939). Primers and TaqMan™ probes were designed using the Primer Express software package (Applied Biosystems, Foster City, CA) and synthesized by Applied Biosystems. Each probe was labeled with the reporter dye 6-carboxyfluorescein (FAM) at the 5' end, with the quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. The probe was phosphate-blocked at the 3' end to prevent extension by AmpliTaq Gold DNA polymerase. Primer sequences were as follows: for medaka cytochrome p450arom forward primer fmp450-267f 5'-CAACAAATATGGAGACATTGTTCGA- 3'; reverse primer fmp450-385r 5'-GCGAGGTGTATTTTCTGTTCTTCTTGA-3', and the TaqMan™ probe fmp450-325p 5-6-FAM- CTGATCCTCAGCAGGGCATCAGCAGTG-TAMRA-3;
For the medaka estrogen receptor; forward primer fmOesR-835f 5'-GTTGTAAAGGGTCAGGAGCATAAAAC-3', reverse primer fmOesR-1041r 5'-CATCATGGTGACCTCGGTGTAC-3' and the TaqMan™ probe fmOesR-943p 5-6-FAM-CCTCCTGAGCAGGTGCTGCTCCTCCT-TAMRA-3.

Quantitative analyses of medaka cDNA were performed in comparison to an endogenous control (18S rRNA TaqMan™ system, Applied Biosystems), and were run in separate wells. TaqMan™ PCR reaction solutions consisted of 400nM of each primer, 80 nM of the specific TaqMan™ probe, and TaqMan™ Universal PCR Mastermix (Applied Biosystems) containing 10mM Tris-HCl (pH 8.3), 50 mM KCl, 5mM Mg Cl_2, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase, 0.25 U AmpErase UNG, and 5 µl of the cDNA sample. PCR reaction solutions were placed in 96-well plates and amplified in an automated laser-based fluorometer that performs both the thermal cycling and fluorescence detection (ABI PRISM™ 7700 Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 60 sec at 60°C. Performance of each TaqMan system was tested by determination of amplification efficiency (2-fold diluted cDNA preparations were used to obtain standard curves), and sequencing PCR products.

Final quantitation of PCR products was calculated using Sequence Detection Systems (SDS) software version 1.6.3 (Perkin Elmer Biosystems, Foster City, CA) and the Comparative C_T method of calculation (ABI Prism, 1997; Leutenegger, et al., 1999). SDS-PCR amplification plots record FAM-generated fluorescence during amplification. Measurements are taken from the exponential range of the reaction, where reactant concentrations are not limiting. Detection of PCR products is measured as an increase in fluorescence over time, where fluorescence is proportional to the quantity of product (Leutenegger, et al., 1999; Leutenegger, 2001). In an amplification plot, which represents the increase in FAM-produced fluorescence plotted against the number of thermal reaction cycles, the intersection of the reaction curve and the threshold (where the threshold is defined as 10 times the standard deviation of the background

fluorescence intensity) is referred to as the cycle threshold, or C_T value. [Background fluorescence intensity was measured between cycle 3 and 15 (Leutenegger, 2001).] The C_T value is directly related to the amount of PCR DNA product, and thus is also directly related to the initial amount of target mRNA present in sample tissue. Results are reported as the n-fold difference in transcription relative to control cDNA after normalization of the transcript signals to the endogenous control 18S rRNA.

Statistical analyses

Statistical significance was calculated with NCCS statistical software using the nonparametric Kruskal Wallis test for one-way ANOVA. That test was used to compare differences in the median relative levels of p450arom mRNA and ER mRNA values between individual treatment groups and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons, such that $p < 0.008$ represents a result that is significantly different from controls. Linear regression analysis was used to test for the presence of correlations between relative levels of p450arom mRNA and ER mRNA within tissues.

Results

Both Surflan™ and oryzalin affected the expression of ER mRNA and of p450arom mRNA. Those effects are gender and tissue specific, and depend on the duration of exposure.

24h Exposure

Short-term exposure of medaka to Surflan™ and oryzalin had an apparent inhibitory effect on whole brain ER mRNA (Table 3-1). Although none of the treatment groups exhibited ER

mRNA levels that were significantly different from controls ($p < 0.008$), a number of the treated groups had ER mRNA levels that approached statistical significance ($p < 0.05$). In treated medaka, regardless of gender (F+M), the apparent inhibition of ER mRNA became more pronounced with increasing dose for all treatments of either Surflan™ or oryzalin, with high- and mid-dose groups approaching significance for both compounds. Treated males, and to a lesser extent treated females, also exhibited a consistent, albeit non-significant inhibition of ER mRNA. Males in the low- and mid-dose groups of both Surflan™ and oryzalin had levels of ER mRNA that neared statistical significance when compared to controls.

The response of gonadal ER mRNA to Surflan™ and oryzalin was not linearly related to dose (Table 3-2), and in F+M, or in females treated with either Surflan™ or oryzalin, or males exposed to oryzalin, there was some indication of an "inverted U" shaped dose-response relationship (see Discussion section and Melnick et al., 2001). Both Surflan™ and oryzalin tend to induce expression of gonadal ER mRNA, with statistically significant induction occurring in mid-dose Surflan™ (F+M), and high- and low-dose oryzalin (F+M). Females in the low dose Surflan™ and oryzalin groups and males in all oryzalin treatments also exhibited significant induction of ER mRNA.

Hepatic ER mRNA was strongly induced by both Surflan™ and oryzalin, with statistically significant effects in all F+M groups (Table 3-3). There was also very pronounced induction of hepatic ER mRNA in males or females evaluated separately. ER mRNA levels were statistically higher than controls in both genders and all groups except low dose oryzalin, where effects approached significance.

No significant effects on whole brain p450arom mRNA were observed after 24h exposures to Surflan™ or oryzalin, and there were no consistent effects of chemical, dose, or gender (Table 3-4). However, certain doses were associated with responses that appear noteworthy e.g., high-dose Surflan™ elicited a nearly 145-fold increase in brain p450arom mRNA in females. Low-dose Surflan™ males, and both males and females exposed to high-dose oryzalin exhibited a more modest apparent induction of brain p450arom mRNA.

Expression of gonadal p450arom mRNA was increased by exposure to Surflan™ or oryzalin, with statistically significant effects documented in all oryzalin groups (F+M), and mid-dose Surflan™ F+M (Table 3-5). Gender-specific effects also reached significance in mid-dose oryzalin females and in males in all oryzalin treatment groups.

Hepatic p450arom mRNA was not detected in controls, or in low-dose oryzalin animals of either gender in 24h samples. F+M in the two highest Surflan™ groups and in the mid-dose oryzalin group exhibited significant induction of hepatic p450arom mRNA, and low-dose Surflan™ and high-dose oryzalin responses neared significance (Table 3-6). In males, hepatic p450arom mRNA levels were significantly elevated in all groups where it was detected.

Linear regression analysis was used to determine whether relative levels of p450arom mRNA were correlated with ER mRNA in a given tissue. For 24h samples, there was a strong relationship between p450arom and ER mRNAs in gonads: F+M ($r^2 = 0.39$, $p = 0.0000$); females ($r^2 = 0.23$, $p = 0.02$); and males ($r^2 = 0.52$, $p = 0.0002$). A significant relationship was also

evident in two of three groups in the liver: F+M ($r^2 = 0.21$, $p = 0.003$); and males ($r^2 = 0.69$, $p = 0.000000$). No other tissues or treatment groups displayed a significant relationship between the two mRNAs in the 24h sample.

48h Exposure

After 48h of exposure, whole brain ER mRNA tended to remain at lower levels in exposed groups than in control animals, and was significantly lower in females in the mid- and low-dose groups exposed to either Surflan™ or oryzalin (Table 3-7). Exceptions to this are seen in both high-dose Surflan™ and high-dose oryzalin males, who exhibited higher, although non-significant levels of brain ER mRNA than controls. The induction of gonadal ER mRNA seen in most treatment groups after 24h exposure had largely changed to inhibition by 48h (Table 3-8). F+M exhibited significant inhibition of ER mRNA in high-dose Surflan™ as well as high- and mid-dose oryzalin groups. Although ovarian and testicular ER mRNA levels were inhibited relative to controls in four and five groups respectively, these effects did not reach statistical significance.

In many treatment groups, hepatic ER mRNA levels had returned to near-control levels by 48h, although levels remained elevated (albeit not significantly) in females exposed to high- and low-dose Surflan™, high- and mid-dose oryzalin, and in males exposed to low-dose oryzalin (Table 3-9). Hepatic ER mRNA in males and females (combined) were also elevated in these same treatment groups.

Whole-brain p450arom mRNA levels had largely equilibrated by 48h, and exhibited none of the occasional marked elevations observed at 24h (Table 3-10). Liver p450 arom mRNA was not

detected in any medaka sampled at 48h, including controls. These initial results were confirmed by re-analysis of all 48h liver samples (see Discussion section). Gonadal p450arom mRNA levels were markedly lower than controls in many 48h samples. However, there were clear exceptions to this, with levels above those seen in controls, in low-dose Surflan™ and high-dose oryzalin females, and mid-dose Surflan™ and high-dose oryzalin males. None of the gonadal p450arom mRNA levels were significantly different from controls (Table 3-11).

Relative levels of p450arom mRNA and ER mRNA were not correlated in any 48-h tissue or treatment group.

21d Exposure

No consistent effects of exposure were apparent in levels of whole brain ER mRNA after 21 days, although there were significantly elevated levels of ER mRNA in F+M and males exposed to high-dose oryzalin, and levels of whole brain ER mRNA neared statistical significance in high-dose oryzalin females (Table 3-12). No treatment-related effects on gonadal ER mRNA were discernable in any exposure group at 21 days (Table 3-13). Hepatic ER mRNA levels in females were generally lower than in controls, while males tended to have somewhat higher hepatic ER mRNA levels in 21-d samples. No gender-specific responses in treatment groups were statistically different from control values (Table 3-14).

Levels of whole brain p450arom varied widely in fish exposed for 21d. Mean relative p450arom mRNA levels of control animals were very low for this time-point, as samples from a number of males and females had no detectable p450arom mRNA. By comparison, whole brain p450arom mRNA levels were elevated in the mid- and low-dose Surflan™ F+M groups, high-dose oryzalin

F+M group, as well as in the corresponding gender-specific groups. However, effects were significant only in the high-dose oryzalin group of males and females considered together, and females considered separately (Table 3-15).

At 21d, gonadal p450arom mRNA remained lower than controls in all male treatment groups; these results approached statistical significance in males from the mid- and low-dose Surflan™ groups (Table 3-16). In females, no consistent response of p450arom mRNA to exposure was apparent. Hepatic p450arom mRNA was not detected in 21d samples from male and female medaka exposed to low-dose Surflan™, or males exposed to high-dose Surflan™, and only very low levels were detected in mid-dose oryzalin males. In all other groups, treatment was consistently associated with an apparent induction of p450arom mRNA in medaka exposed to either Surflan™ or oryzalin (Table 3-17). Nonetheless, these effects were not significantly different than controls. In the oryzalin groups in particular, our inability to demonstrate statistical significance may be largely attributable to the marked variability in the magnitude of individual responses.

P450arom mRNA and ER mRNA levels were not significantly associated in any tissue in medaka exposed to Surflan or oryzalin for 21 d.

Discussion

Surflan™ and oryzalin affected expression of ER mRNA and p450arom mRNA in the critical reproductive tissues of brain, gonad, and liver. Although individual variability and small sample

size limited our ability to discern effects, it is clear that in medaka, Surflan™ and oryzalin affect regulation of mRNA of these two important proteins.

P450arom is responsible for the conversion of aromatizable androgens to estrogens in vertebrate brain and gonadal tissue (see reviews of Lephart, 1996; Conley and Hinshelwood, 2001). As such, p450arom activity is an important regulator of estrogen concentrations, and is critical to estrogen-mediated biological processes. Because changes in p450arom mRNA correspond to changes in p450arom activity (Tanaka et al., 1992; Fukada et al., 1996; Chang et al., 1997; Gen et al., 2001), our data suggest that levels of p450arom mRNA, aromatase enzymatic activity, and thus synthesis of 17-β estradiol may be altered, sometimes dramatically, by exposure to Surflan™ and oryzalin. Similarly, because changes in ER mRNA expression are known to correspond to changes in ER number and binding capacity (Pakdel et al., 1991; Flouriot et al., 1993; Mackay and Lazier, 1993), it is possible that Surflan™ and oryzalin affect the expression of estrogen-regulated genes. The ability of Surflan™ and oryzalin to alter estrogen-regulated gene expression is consistent with their effects on the high molecular weigh choriogenins described in Section 1.

Our data indicate that exposure to Surflan™ and oryzalin is associated with a rapid (within 24h) induction of both gonadal and hepatic p450arom and ER. Although we were not able to measure plasma estrogen levels, these effects are consistent with a tissue-specific increase in p450arom-mediated synthesis of estrogen, and an estrogen-stimulated increase in ER; Pakdel et al. (1991) observed induction of ER and ER mRNA in parallel within a few hours of administration of a single dose of estradiol to rainbow trout. The apparent but non-significant inhibition of whole

brain ER mRNA at 24h may be a response to the postulated increase in circulating levels of estrogen that originate in the liver and/or gonads. Although this type of negative feedback has not been specifically documented in teleosts, ER's are present in the pars distalis of teleost pituitary where the gonadotroph cells are located (Salbert et al., 1991) and steroid feedback effects on the hypothalamic-pituitary-gonadal axis have been reported (Trudeau et al., 1991; Trudeau et al., 1993).

The existence of hepatic ERs and responsiveness of hepatic ERs to estrogen and xenoestrogens is well documented in teleosts (Mommsen and Lazier, 1988; Pakdel et al., 1991; Mackay and Lazier, 1993; Salbert et al., 1993; Mackay et al., 1996; Yadetie et al., 1999). However, hepatic p450arom is poorly characterized in both mammals (Biegel et al., 1995; Liu et al., 1996; You et al., 2001) and teleosts, with investigations that have explicitly sought evidence of hepatic p450arom in teleosts failing to detect it (Gelinas et al., 1998; Kwon et al., 2001). Those investigations - as well as ours - used probes based on ovarian p450arom. Although the generally robust response in liver that we documented after a 24-h exposure suggests that the ovarian isoform of aromatase is expressed in medaka liver, there were notable exceptions (e.g., our inability to detect p450arom in either controls or low-dose oryzalin animals of both genders at this time point). At the present time, we do not know if the variability in response is partially or completely attributable to the presence of a non-ovarian isoform of p450arom that is imperfectly detected by our probe, or whether our measurements simply reflect individual variability.

The absence of a clear effect on brain p450arom mRNA was unexpected in light of recent data that indicate the activity of brain p450arom was increased in medaka exposed to exogenous estrogen (Melo and Ramsdell, 2001), and in feral mosquitofish exposed to papermill effluent (Orlando et al., 2002). Furthermore, both estrogen and the xenoestrogen bisphenol A caused an elevation of p450arom mRNA in the brain of zebrafish (Kishida et al., 2001). One explanation of our results may be that medaka have an as yet-unidentified p450arom isoform specific to brain that is either not detected or not efficiently detected by the ovarian p450arom probes that we used. If this is the case, then our results also suggest that ovarian p450arom in brain is largely non-responsive to Surflan™ and oryzalin. The existence of a single p450arom gene is suggested by data from studies in mammals (Simpson et al., 1997), medaka (Tanaka et al., 1995), and fathead minnow (Halm et al., 2001). However, the presence of multiple, closely related p450arom isoforms have been reported in pigs (Choi et al., 1997), goldfish (Tchoudakova and Callard, 1998), zebrafish (Kishida et al., 2001), Nile tilapia (Kwon et al., 2001), the Mozambique tilapia (Cruz and Canario, 2000), and suggested by results in the European sea bass (Dalla Valle et al., 2002). In goldfish, where distinct ovarian and brain p450arom genes have been identified, Northern blot analysis using cDNA of the brain isoform did not detect p450arom transcript in ovary, testis, or liver (Gelinas et al., 1998). In Nile tilapia, both brain p450arom and ovarian p450arom were expressed in brain and gonads, although the brain isoform was expressed at highest levels in the brain, and the ovarian isoform was expressed at highest levels in the ovaries (Kwon et al., 2001). Additionally, low levels of ovarian p450arom in the brain of European sea bass were deemed inconsistent with the high levels of p450arom typically reported in teleost brain (Callard and Pasmanik, 1987), suggesting the possibility of a brain-specific isoform in this species as well (Dalla Valle et al., 2002).

After 48h of exposure, a dramatic reversal in gonadal p450arom mRNA expression occurred. The marked induction of p450arom mRNA in gonads at 24h changed to an apparent inhibition of p450arom mRNA in most dose groups, although this effect was not significantly different from controls. The apparent reversal in p450arom mRNA expression was not seen uniformly e.g., mid-dose Surflan™ and high-dose oryzalin males, and low-dose Surflan™ females continued to express higher levels of p450arom mRNA than controls. However, even in these three dose groups, the decline in p450 arom mRNA was dramatic when compared to p450mRNA levels in these same treatment groups at 24h. Gonadal ER mRNA levels tended to mirror the response in p450arom mRNA, exhibiting a marked change from enhanced expression relative to controls at 24h, to a marked inhibition at 48h. Although statistical significance of these effects was achieved only in the F+M groups (Surflan™ high-dose, and oryzalin high- and mid-dose), the tendency for down-regulation of gonadal ER mRNA appears to be a real phenomenon.

Neither whole brain p450 arom mRNA or ER mRNA in treated animals was significantly different from controls after 48h exposure. However, in females, there was a tendency for brain p450arom mRNA levels to have declined relative to the mRNA values measured at 24h; a similar effect was not apparent in males.

The induction of hepatic ER mRNA seen at 24h was no longer evident in 48h samples. Although some modest elevation in expression of ER mRNA, relative to controls, was seen in females of most treatments, and was dramatic in high-dose oryzalin females, the effects were not statistically significant. Unfortunately, the hepatic ER mRNA response cannot be interpreted

within the context of hepatic p450arom mRNA levels, since we were not able to detect p450arom mRNA in any liver samples from this time point. As with the 24h liver samples, cDNA from all of these tissues was reanalyzed, and confirmed the original results. We do not know if the apparent absence of hepatic p450arom mRNA is due to the presence of a non-ovarian form of p450arom in the liver that is not consistently or efficiently detected by the ovarian p450arom probe, or to some other phenomenon that is not currently understood. Certainly, the consistency of the results at this point in time seems to exclude the possibility of individual variation as an underlying explanation.

After 21d of exposure to Surflan™ and oryzalin, there was no consistent effect on whole brain p450arom mRNA or ER mRNA, nor was there a readily discernable difference in levels of these mRNAs between samples analyzed at 48h and 21d. It is interesting to note however, that the sole treatment group to display a significant effect on whole brain ER mRNA at 21 d (high-dose oryzalin, all gender groupings) also tended to exhibit a significant elevation in whole brain p450arom mRNA. Gonadal ER mRNA levels were generally higher in both females and males at 21d relative to 48h, but there were no obvious or statistically significant effects between treatments. Although males in all exposure groups tended to have lower levels of gonadal p450arom mRNA than controls, this effect neared significance only in low- and mid-dose Surflan™. Gonadal p450arom mRNA levels in females exposed for 21d were not significantly different from controls.

At 21d, hepatic ER mRNA levels in treated females were lower than controls, and were also lower than hepatic ER mRNA measured after 48h exposure. Males had higher but non-

significant levels of ER mRNA when compared to controls. These levels were also higher relative to male hepatic ER mRNA measured at 48h.

Expression of hepatic p450arom mRNA in 21d samples was characterized by apparently dramatic induction in many treatment groups, with the most notable levels occurring in oryzalin-treated animals. Individual responses varied widely however, and in combination with small sample size prevented demonstration of statistical significance. High-dose Surflan™ males and all low-dose Surflan™ animals had no detectable hepatic p450arom at this time point. Nonetheless, the data in Table 3-17 indicate that a 21d exposure to Surflan™ or oryzalin may have substantial effects on hepatic p450arom, but that these effects vary dramatically with the dose and with the individual.

It is clear that the response of medaka to Surflan™ and oryzalin is characterized by considerable variability. At present, the underlying cause(s) of this variability are unknown, but may be generally attributable to the outbred nature of this strain of medaka (Davis, 2001) and thus may simply reflect the normal variation found in populations of feral fish. Other likely sources of variabiality include differences in uptake, metabolism, or elimination of Surflan™ and oryzalin. To the best of our knowledge, none of these pharmacokinetic parameters have been studied for Surflan™ and oryzalin in any species – either teleost or mammalian. Consequently, what contribution each paramater might introduce to an individual's response is entirely unknown. In addition, although concentrations of Surflan™ were selected to yield doses of oryzalin approximately equal to those in each of the oryzalin treatment groups, Surflan™ is an emulsion, and contains petroleum hydrocarbons of unspecified identity. The characteristics of an emulsion

and the presence of the petroleum hydrocarbons may potentially affect uptake and distribution of the oryzalin contained in Surflan™. The petroleum hydrocarbons may also elicit some toxicity on their own or modulate oryzalins' effects.

One of the fundamental premises of classical toxicology is that response is directly related to exposure concentration and duration. That relationship may not apply to the biochemical processes that mediate the toxicity of xenoestrogens in general (Melnick et al., 2002), and to Surflan™ and oryzalin in particular. Indeed, there are many examples in our data where a linear dose-response relationship does not exist. That a non-linear dose-response relationships may exist for ER mRNA and p450 mRNA is not surprising considering the complexity and potential tissue-specific nature of the regulatory processes governing expression of these mRNAs. Indeed, the responses of ER mRNA and p450mRNA seem not to be related to dose over time – but instead, seem to be dynamic within the first 48h of exposure, with a tendency to equilibrate some time between 48h and 21d. (The one apparent exception to this is hepatic p450arom, which exhibited a tendency to respond to exposure by enhanced expression at 21-d.) It is likely that the apparent non-linearity in dose-response, as well as the processes of equilibration of ER and p450 mRNAs, are sources of some of the considerable variability present in our data. Specifically how these factors contribute to that variability are not understood at the present.

One of our hypotheses was that a tissue-specific change in p450arom mRNA (and the presumed change in estrogen synthesis) would lead to a corresponding effect in ER mRNA expression in the same tissue. Expression of p450arom mRNA and ER mRNA appeared to be affected by treatment in a qualitatively similar manner in 24h-gonads, and this was confirmed by regression

analysis. Twenty four-hour liver samples of the F+M grouping and males evaluated separately also displayed a significant correlation between p450arom mRNA and ER mRNA levels. No other statistically significant correlations were found between the relative levels of these two mRNAs. The absence of a correlation between expression of p450arom mRNA and ER mRNA in most tissues and/or time points may be due in part to limitations in our ability to detect correlations because of small sample size and marked variability in response. However, neither the temporal sequence between induction of p450arom mRNA and ER mRNA or the tissue-specific relationship between these mRNAs has been explored in any species. It is probable that there are associations between the expression of the two mRNAs that are more complex than could have been detected by our experimental design. Indeed, the complex regulation of p450arom and the likelihood of regulatory feedback loop involvement in the expression of both mRNAs suggests that aspects of regulation of one or both of these mRNAs may occur outside of the affected tissue. Consequently, although correlations may exist beyond the few that we detected, detection of those additional correlations may require measurements of biochemical endpoints beyond those evaluated here.

That a complex relationship exists between p450arom, levels of endogenous estrogen, and the ER is suggested by the different regulation strategies of p450arom. In ovarian tissue, estrogen-mediated regulation of p450arom is indirect. Based on recent mammalian studies, induction of p450arom gene expression in ovarian granulosa cells occurs in response to the gonadotropin follicle stimulating hormone (FSH). That response is mediated by a cAMP-signaling cascade and phosphorylation of transcription factors such as CREB (Fitzpatrick et al., 1993) and SF-1 (Lala et al., 1992; Morohashi et al., 1992). In reproductively inactive fish, aromatizeable

androgens and estrogens act to enhance GtH mRNA accumulation in the pituitary (Counis et al., 1987). In reproductively active fish, such as the medaka used in this study, estrogen acts by negative feedback at the pituitary to suppress gonadotropins (Billard et al., 1977). P450arom activity can be induced *in vitro* in teleosts by incubating early-vitellogenic follicles with GtHs or drugs that increase intracellular cAMP (Kagawa et al., 1984; Nagahama et al., 1991). Tanaka et al. (1995) have identified sequences in the promoter region of the medaka ovarian p450arom gene that are identical to the transcription factor Ad4 binding protein (Ad4BP, also known as steroid factor-1, SF-1), a steroid receptor-like protein found in the promoter regions of other p450arom genes (Morohashi et al., 1992). Tanaka et al. (1995) also identified two sequences that correspond to the latter half of an estrogen response element (ERE) on the medaka p450arom gene, and raised the possibility that there is a steroid-hormone receptor-like protein that regulates p450arom transcription by recognizing both the Ad4BP/SF-1 and ERE sequences. Based on this current understanding, both estrogen and xenoestrogens act on ovarian p450 indirectly via action at the pituitary, but this response may be modified by receptor binding to the Ad4BP/SF-1 and ERE in tissues containing the ovarian isoform of p450arom.

Regulation of p450arom in the brain of mammals is distinct from that in gonadal tissue (Roselli and Resko, 1993). Characterization of the promoter regions of human p450arom have identified at least six different promoters, thought to be responsible for the tissue-specific expression of p450arom in humans (Means et al., 1991; Harada et al., 1993). In those species of teleosts where brain-specific forms of p450arom have been identified and sequenced, the presence of multiple ERE's suggests a direct regulatory action of estrogen (Gelinas et al., 1998; Callard et al., 2001). That estrogen directly regulates p450arom in brain is also indicated by the observation that

estrogen production of primary cultures of goldfish forebrain or pituitary was regulated by estrogen concentration in the culture media (Callard and Pasmanik, 1987). Additionally, treatment with exogenous estrogen increased brain p450arom activity in goldfish and medaka (Callard et al., 1995; Melo and Ramsdell, 2001).

Cell sensitivity to estrogen and xenoestrogens is typically the result of interaction of these ligands with the ER. Ligand-activated ER is a transcription factor that regulates gene expression directly by binding as a homodimer to an ERE, or indirectly by interaction with other transcription factors (Glass et al., 1997; Moras and Gronomeyer, 1998). In many species, the ER consists of at least two isoforms, ERα and ERβ; separate genes that have distinct tissue distributions and ligand binding characteristics (Paech et al., 1997; Kuiper et al., 1997; Tchoudakova et al., 1999; Menuet et al., 2001; Wu et al., 2001). That ERα and ERβ have different binding activities for certain ligands, suggests a different regulatory function for the different receptor subtypes (Paech et al., 1997). In teleosts, an ERα has been identified in rainbow trout (Pakdel et al., 1989), tilapia (Tan et al., 1995), channel catfish (Xia et al., 1999), and Japanese eel (Todo et al., 1996). An ERβ subtype is known from gilthead sea bream (Socorro et al., 2000), channel catfish (Xia et al., 2000), and goldfish (Tchoudakova et al., 1999). A third ER type, ERγ (or ERβ2) has been reported in the Atlantic croaker (Hawkins et al., 2000) and goldfish (Ma et al., 2000). In medaka, only a single ER has been identified to date; that receptor, sequenced from hepatic tissue, has not been characterized as to its specific subtype (Kawahara and Yamashita 1999).

Estrogen up-regulates hepatic ER in all oviparous species studied to date (Lazier et al., 1985; Barton and Shapiro, 1988; MacKay and Lazier, 1993; Pakdel et al., 1991; Salbert et al., 1993; Mackay et al., 1986). In *Xenopus* and rainbow trout, estrogen-induced Vg mRNA induction is preceded by an increase in ER level (Barton and Shapiro, 1988; Pakdel et al., 1991; Mackay and Lazier, 1993; Flouriot et al., 1995). The magnitude of estrogen-induced ER mRNA induction is comparable between species, and the transcriptional response of the Vg gene is directly proportional to the amount of ER (Flouriot et al 1997). Nonylphenol-induced ER mRNA synthesis preceded both Vg and Cg mRNA synthesis in Atlantic salmon liver (Yadetie et al., 1999).

The dramatic induction of ER mRNA in liver and gonads of Surflan^{TM} - and oryzalin-exposed animals suggests that oryzalin alone or oryzalin present in Surflan^{TM} is perceived biologically as an estrogen in these tissues. If trout and salmon Vg and Cg genes are considered models for medaka, our data suggest that up-regulation of the ER precedes the estrogen-mediated induction of other genes – as yet unidentified – in medaka liver, ovaries, and testes. In rainbow trout, both ER and Vg gene induction persisted for approximately two weeks in response to a single challenge with estrogen (Pakdel et al., 1991). Barton and Shapiro (1988) reported the permanent induction of ER in *Xenopus* following estrogen treatment. This phenomenon does not seem to hold for medaka exposed to Surflan or oryzalin, as the induction of gonadal and hepatic ER seen at 24h had largely reversed by 48h, and showed some indication of continued down-regulation at 21d.

Whereas circulating levels of estrogen regulate hepatic and gonadal ER in teleosts, regulation of brain ER is differentially affected by circulating estrogen or by estrogen synthesized by p450arom in neural tissues from aromatizeable androgens (Pasmanik and Callard, 1988; Callard et al., 1995). In goldfish, the brain isoform of p450arom contains EREs, indicating the likelihood that estrogen directly regulates expression of this enzyme via classical ER/ERE interactions (Callard et al., 2001). Tissue-specific differences in receptor subtype, distribution, or number, may also influence the response to estrogen (Kuiper et al., 1997; Paech et al., 1997). Tchoudakova et al. (1999) have cloned an ERβ subtype from goldfish liver; using ERβ cDNA, several variants of ER mRNA were detected in goldfish liver and ovary, but expression was below detection in brain. The ER mRNA response in medaka brain to Surflan™ and oryzalin was minimal as detected by our ER probes. Those probes were based on medaka liver ER, the only ER sequenced to date in this species (Kawahara and Yamashita 1999). As with brain p450arom mRNA, it appears possible that there is a different ER subtype in medaka brain than in liver, and that the brain subtype is poorly detected by the hepatic ER probe. Alternatively, medaka brain and liver may express the same ER subtype, and Surflan™ and oryzalin may simply have little effect on brain ER.

Conclusions

We have demonstrated that the xenoestrogens Surflan™ and oryzalin have significant effects on the gonads and liver. A 24h exposure to Surflan™ and oryzalin induced dramatic increases in gonadal and hepatic p450arom mRNA and ER mRNA. These effects largely reversed at 48h, and 21d, there was little evidence that these mRNAs were affected by exposure. An exception to this was seen in hepatic p450arom mRNA from 21d samples. Although the response was

extremely variable and not statistically significant, our results suggest that Surflan™ and oryzalin continued to affect expression of the p450arom gene in liver. Few effects of Surflan™ or oryzalin were observed on whole brain ER mRNA and p450arom mRNA at any time during the experiment. We hypothesize that there may be brain-specific forms of the ER and p450arom genes that are poorly detected by our probes. Alternatively, if the respective liver ER and ovarian p450arom mRNAs are present in brain, they are minimally responsive to Surflan™ and oryzalin.

The documented persistence in ER induction following a single dose of estrogen (Pakdel et al., 1991; Mackay and Lazier, 1993) does not correspond to the effects on hepatic and gonadal ER mRNA in medaka continuously exposed to Surflan™ and oryzalin. Instead, an apparent compensatory down-regulation of ER mRNA began between 24 and 48h and was maintained to 21d. Two of the critical questions raised by this response are (1) what effect does the initial up-regulation of ER, or the ensuing down-regulation of ER have on gene transcription; and (2) do effects on transcription or transcription products continue once ER equilibration has taken place? Understanding the dynamics of p450arom-generated estrogen are also integral to answering these questions; specifically, whether the initial increase in hepatic and gonadal p450arom and the presumed associated increase in estrogen have persistent effects, or whether effects are transitory, normalizing as p450arom mRNA equilibrates? The apparent persistence of effects on hepatic p450arom in certain treatment groups after 21d of exposure introduces a number of questions concerning testosterone and estrogen homeostasis. As the primary substrate for p450arom and a metabolic precursor to estrogen, testosterone levels are regulated by estrogens' action on 17-α hydroxylase, C17/20 lyase (Dufau, 1988) and p450arom (Kagawa et al., 1984;

Nagahama et al., 1991; Tanaka et al., 1995; Callard and Pasmanik, 1987; Callard et al., 2001). How circulating levels of testosterone and estrogen are affected by sustained induction of hepatic p450arom is entirely unknown at this time.

In summary, the data described here represent the results of an initial examination of the effects of p450arom mRNA and ER mRNA following exposure to Surflan™ and oryzalin. Our data demonstrate that Surflan™ and oryzalin elicit a spectrum of biological effects in medaka, and do so after exposure to the same concentration of oryzalin that impaired reproduction and caused gonadal lesions following a 21d exposure. Although many questions remain concerning the mechanistic basis of Surflan™ and oryzalins' toxicity, our results suggest that perturbations in estrogen synthesis and ER dynamics may be circumstantially linked to the adverse reproductive effects caused by these compounds.

Table 3-1. Relative Levels of Medaka Whole Brain Estrogen Receptor mRNA after 24h Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative Estrogen Receptor mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	1.23	1.06	1.41
Surflan™ 2.5 µl/l	0.13 *	0.17 [n.s]	0.08 *
Surflan™ 1.3 µl/l	0.18 *	0.25 [n.s]	0.11 *
Surflan™ 0.67 µl/l	0.49 [n.s]	0.18 [n.s]	0.80 [n.s]
Oryzalin 1 mg/l	0.22 *	0.30 [n.s]	0.14 *
Oryzalin 0.5 mg/l	0.24 *	0.27 [n.s]	0.22 *
Oryzalin 0.25 mg/l	0.82 [n.s]	1.60 [n.s]	0.04 *

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 1.48; p = 0.21; DF = 6, 35.
[b] n= 6
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 2.36; p = 0.09; DF = 6, 14.
[d] n= 3
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 0.88; p = 0.54; DF = 6, 14.
[n.s]= not significant.
* $p < 0.05$. Not significant by the criterion for statistical significance ($p < 0.008$) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.

Table 3-2. Relative Levels of Medaka Gonad Estrogen Receptor mRNA after 24h Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative Estrogen Receptor mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	1.2	1.4	1.1
Surflan™ 2.5 µl/l	3.4 [n.s]	0.7 [n.s]	6.2 [n.s]
Surflan™ 1.3 µl/l	1200.0 **	2000.0 *	430.0 *
Surflan™ 0.67 µl/l	290.0 [n.s].	580.0 **	3.4 [n.s]
Oryzalin 1 mg/l	4700.0***	310.0 *	9000.0 **
Oryzalin 0.5 mg/l	38000.0 *	7.8 [n.s].	75000.0 **
Oryzalin 0.25 mg/l	47000.0 ***	14000.0 ***	78815.0 ***

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 11.02; p = 0.000001; DF = 6, 35.
[b] n= 6
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 9.32; p = 0.0003; DF = 6, 14.
[d] n= 3
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 9.10; p = 0.0004; DF = 6, 14.
[n.s]= not significant.
* $p < 0.05$. Not significant by the criterion for statistical significance ($p < 0.008$) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.
** $p < 0.005$
*** $p < 0.001$

Table 3-3. Relative levels of medaka liver estrogen receptor mRNA after 24h exposure to Surflan™ or oryzalin. Values given are arithmetic means; data were log-transformed for analysis. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative Estrogen Receptor mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d.e]
Control	2.26	4.31	0.21
Surflan™ 2.5 µl/l	32000.0 ***	33000.0 ***	30000.0 ***
Surflan™ 1.3 µl/l	340000.0 ***	68000.0 ***	5900.0 ***
Surflan™ 0.67 µl/l	7500.0 ***	5800.0 ***	9200.0 ***
Oryzalin 1 mg/l	140000.0 ***	250000.0 ***	29000.0 ***
Oryzalin 0.5 mg/l	4300.0 ***	1900.0 **	6700.0 ***
Oryzalin 0.25 mg/l	250.0 **	480.0 *	1.0 *

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 18.76; p = 0.000000; DF = 6, 35.
[b] n= 6
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 7.60; p = 0.0009; DF = 6, 14.
[d] n= 3
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 18.5; p = 0.000006; DF = 6, 14.
* $p < 0.05$. Not significant by the criterion for statistical significance ($p < 0.008$) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.
** $p < 0.005$
*** $p < 0.001$

Table 3-4. Relative Levels of Medaka Whole Brain p450Aromatase mRNA after 24h Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative p450 mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	1.02	1.02	1.02
Surflan™ 2.5 µl/l	73.19 [n.s]	144.94 [n.s]	1.43 [n.s]
Surflan™ 1.3 µl/l	1.26 [n.s]	1.65 [n.s]	0.88 [n.s].
Surflan™ 0.67 µl/l	7.40 [n.s]	1.02 [n.s]	13.77 *
Oryzalin 1 mg/l	2.82 [n.s]	3.17 [n.s]	2.48 [n.s]
Oryzalin 0.5 mg/l	1.28 [n.s].	1.42 [n.s]	1.15 [n.s]
Oryzalin 0.25 mg/l	0.32 [n.s]	0.24 [n.s]	0.39 [n.s]

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 0.98; p = 0.45; DF = 6, 35.
[b] n= 6
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 1.0; p = 0.46; DF = 6, 14.
[d] n= 3
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 1.34; p = 0.30; DF = 6, 14.
[n.s] = not significant.
* $p < 0.05$. Not significant by the criterion for statistical significance ($p < 0.008$) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.

Table 3-5. Relative Levels of Medaka Gonad p450Aromatase mRNA after 24h Exposure to Surflan™ or Oryzalin. Values given are arithmetic means; data were log-transformed for analysis. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative p450 mRNA Levels		
	Females and Males [a,b]	Females[c.d]	Males[d,e]
Control	1.36	1.13	1.59
Surflan™ 2.5 µl/l	2.7 [n.s]	1.3 [n.s]	4.1 [n.s]
Surflan™ 1.3 µl/l	630.0 **	780.0 [n.s]	490.0 *
Surflan™ 0.67 µl/l	180.0 [n.s]	360.0 *	1.4 [n.s]
Oryzalin 1 mg/l	15000.0 ***	3.3 [n.s]	30000.0 ***
Oryzalin 0.5 mg/l	170000.0 ***	330000.0 ***	4200.0 ***
Oryzalin 0.25 mg/l	3700.0 ***	140.0 *	7200.0 ***

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 9.20; p = 0.000005; DF = 6, 35.
[b] n= 6
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 6.16; p = 0.0025; DF = 6, 14.
[d] n= 3
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 9.41; p = 0.0003; DF = 6, 14.
[n.s]= not significant.
* $p < 0.05$. Not significant by the criterion for statistical significance ($p < 0.008$) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.
** $p < 0.005$
*** $p < 0.001$

Table 3-6. Relative Levels of Medaka Liver p450Aromatase mRNA after 24h Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative p450 mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	0.0	0.0	0.0
Surflan™ 2.5 µl/l	1100.0 **	2100.0 [n.s]	120.0 ***
Surflan™ 1.3 µl/l	270.0 ***	120.0 [n.s]	430.0 ***
Surflan™ 0.67 µl/l	9.8 *	4.9 [n.s]	180.0 ***
Oryzalin 1 mg/l	7.3 *	4.1 [n.s]	140.0 ***
Oryzalin 0.5 mg/l	680.0 ***	630.0 [n.s]	780.0 ***
Oryzalin 0.25 mg/l	0.0 [n.s]	0.0 [n.s]	0.0 [n.s]

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 4.36; p = 0.002; DF = 6, 35.
[b] n= 6
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 1.54; p = 0.24; DF = 6, 14.
[d] n= 3
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 12.74; p = 0.000057; DF = 6, 14.
[n.s]= not significant.
p < 0.05. Not significant by the criterion for statistical significance (p < 0.008) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.
** p < 0.005
*** p < 0.001

Table 3-7. Relative Levels of Medaka Whole Brain Estrogen Receptor mRNA after 48h Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative Estrogen Receptor mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	1.8	2.0	1.5
Surflan™ 2.5 µl/l	1.7 [n.s]	0.5 [n.s]	2.8 [n.s]
Surflan™ 1.3 µl/l	0.6 [n.s]	0.2 **	1.1 [n.s]
Surflan™ 0.67 µl/l	0.2 [n.s]	0.2 **	0.2 [n.s]
Oryzalin 1 mg/l	1.3 [n.s]	0.3 *	2.2 [n.s]
Oryzalin 0.5 mg/l	0.1 [n.s]	0.2 **	0.1 [n.s]
Oryzalin 0.25 mg/l	0.2 [n.s]	0.1 **	0.3 [n.s]

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, XX; p = XX; DF = XX
[b] n= 6
[c] Kruskall-Wallace ANOVA results for Females: F ratio, XX; p = XX; DF = XX
[d] n= 3
[e] Kruskall-Wallace ANOVA results for Males: F ratio, XX; p = XX; DF = XX
[n.s]= not significant.
$p < 0.05$. Not significant by the criterion for statistical significance ($p < 0.008$) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.
** $p < 0.005$

Table 3-8. Relative Levels of Medaka Gonad Estrogen Receptor mRNA after 48h Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative Estrogen Receptor mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	1.1	1.2	1.1
Surflan™ 2.5 µl/l	0.0018 **	0.00024 [n.s]	0.0019 [n.s]
Surflan™ 1.3 µl/l	0.25 [n.s]	0.14 [n.s]	0.31 [n.s]
Surflan™ 0.67 µl/l	3.1 [n.s]	2.1 [n.s]	4.1 [n.s]
Oryzalin 1 mg/l	0.017 **	0.053 [n.s]	0.011 [n.s]
Oryzalin 0.5 mg/l	0.025 **	0.035 [n.s]	0.016 [n.s]
Oryzalin 0.25 mg/l	0.00045 [n.s]	--	0.00037 [n.s]

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 2.5; p = 0.041; DF = 6,35
[b] n= 6
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 3.10; p = 0.37; DF = 6,14
[d] n= 3
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 0.97; p = 0.478; DF = 6,14
[n.s]= not significant.
$p < 0.05$. Not significant by the criterion for statistical significance ($p < 0.008$) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.
** $p < 0.005$
-- Not calculated due to loss of 1 sample.

Table 3-9. Relative Levels of Medaka Liver Estrogen Receptor mRNA after 48h Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative Estrogen Receptor mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	1.4	1.2	1.7
Surflan™ 2.5 µl/l	3.6 [n.s]	6.1 [n.s]	1.0 [n.s]
Surflan™ 1.3 µl/l	0.8 [n.s]	1.1 [n.s]	0.4 [n.s]
Surflan™ 0.67 µl/l	4.7 [n.s]	9.4 [n.s]	0.1 [n.s]
Oryzalin 1 mg/l	95.8 [n.s]	191.5 [n.s]	0.1 [n.s]
Oryzalin 0.5 mg/l	2.7 [n.s]	5.2 [n.s]	0.1 [n.s]
Oryzalin 0.25 mg/l	3.7 [n.s]	0.5 [n.s]	5.2 [n.s]

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 0.97; p = 0.461; DF = 6,35
[b] n= 6
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 0.98; p = 0.47; DF = 6,14
[d] n= 3
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 0.75; p = 0.62; DF = 6,14
[n.s]= not significant.

Table 3-10. Relative Levels of Medaka Whole Brain p450Aromatase mRNA after 48h Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative p450 mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	0.9	0.7	1.1
Surflan™ 2.5 µl/l	1.6 [n.s]	0.4 [n.s]	2.8 [n.s]
Surflan™ 1.3 µl/l	0.5 [n.s]	0.5 [n.s]	0.6 [n.s]
Surflan™ 0.67 µl/l	3.8 [n.s]	0.1 [n.s]	0.6 [n.s]
Oryzalin 1 mg/l	2.3 [n.s]	0.4 [n.s]	4.2 [n.s]
Oryzalin 0.5 mg/l	0.6 [n.s]	0.3 [n.s]	0.8 [n.s]
Oryzalin 0.25 mg/l	1.0 [n.s]	0.4 [n.s]	1.6 [n.s]

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 0.45; p = 0.84; DF = 6,14
[b] n= 3
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 0.67; p = 0.68; DF = 6,14
[d] n=3
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 0.80; p = 0.58; DF = 6,14
[n.s]= not significant.

Table 3-11. Relative Levels of Medaka Gonad p450Aromatase mRNA after 48h Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative p450 mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	1.5	1.7	1.4
Surflan™ 2.5 µl/l	0.018 [n.s]	0.036 [n.s]	0.0001 [n.s]
Surflan™ 1.3 µl/l	0.27 [n.s]	0.034 [n.s]	4.2 [n.s]
Surflan™ 0.67 µl/l	5.0 [n.s]	9.9 [n.s]	0.048 [n.s]
Oryzalin 1 mg/l	0.79 [n.s]	1.5 [n.s]	330 [n.s]
Oryzalin 0.5 mg/l	0.0037 [n.s]	0.0017 [n.s]	0.0056 [n.s]
Oryzalin 0.25 mg/l	0.36 [n.s]	0.0025 [n.s]	0.54 [n.s]

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 0.90; p = 0.51; DF = 6,35
[b] n= 6
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 0.93; p = 0.50; DF = 6,14
[d] n= 3
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 2.69; p = 0.06; DF = 6,14
[n.s]= not significant.

Table 3-12. Relative Levels of Medaka Whole Brain Estrogen Receptor mRNA after 21d Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative Estrogen Receptor mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males [d,e]
Control	0.06	0.02	0.1
Surflan™ 2.5 µl/l	0.28 [n.s]	0.49 [n.s]	0.088 [n.s]
Surflan™ 1.3 µl/l	0.28 [n.s]	0.41 [n.s]	1.8 [n.s]
Surflan™ 0.67 µl/l	2.0 [n.s]	2.1 [n.s]	2.0 [n.s]
Oryzalin 1 mg/l	7.8 ***	6.3 *	9.3 **
Oryzalin 0.5 mg/l	0.018 [n.s]	0.028 [n.s]	0.067 [n.s]
Oryzalin 0.25 mg/l	0.034 [n.s]	0.06 [n.s]	0.008 [n.s]

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 6.45; p = 0.00002; DF = 6,76
[b] n=12
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 2.14; p = 0.07; DF = 6,34
[d] n= 6
[e] Kruskall-Wallace ANOVA results for Males: F ratio, XX; p = XX; DF = XX
[n.s]= not significant.
* $p < 0.05$. Not significant by the criterion for statistical significance ($p < 0.008$) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.
** $p < 0.005$
*** $p < 0.001$

Table 3-13. Relative Levels of Medaka Gonad Estrogen Receptor mRNA after 21d Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative Estrogen Receptor mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	1.5	2.4	0.3
Surflan™ 2.5 µl/l	2.0 [n.s]	3.5 [n.s]	0.3 [n.s]
Surflan™ 1.3 µl/l	1.4 [n.s]	2.6 [n.s]	0.3 [n.s]
Surflan™ 0.67 µl/l	1.3 [n.s]	2.7 [n.s]	0.1 [n.s]
Oryzalin 1 mg/l	0.8 [n.s]	1.3 [n.s]	0.2 [n.s]
Oryzalin 0.5 mg/l	0.4 [n.s]	0.5 [n.s]	0.1 [n.s]
Oryzalin 0.25 mg/l	0.8 [n.s]	1.5 [n.s]	0.1 [n.s]

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 0.95; p = 0.47; DF = 6,72
[b] n=12
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 1.06; p = 0.41; DF = 6,33
[d] n= 6
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 1.07; p = 0.40; DF = 6,33
[n.s]= not significant.

Table 3-14. Relative Levels of Medaka Liver Estrogen Receptor mRNA after 21d Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative Estrogen Receptor mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	2.3	3.4	1.1
Surflan™ 2.5 µl/l	0.3 [n.s]	0.4 [n.s]	0.3 [n.s]
Surflan™ 1.3 µl/l	1.0 [n.s]	0.4 [n.s]	1.9 [n.s]
Surflan™ 0.67 µl/l	1.1 [n.s]	0.4 [n.s]	1.8 [n.s]
Oryzalin 1 mg/l	1.8 [n.s]	0.4 [n.s]	3.0 [n.s]
Oryzalin 0.5 mg/l	7.9 [n.s]	0.6 [n.s]	2.5 [n.s]
Oryzalin 0.25 mg/l	1.9 [n.s]	1.0 [n.s]	3.8 [n.s]

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 1.31; p = 0.27; DF = 6,74
[b] n=12
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 2.08; p = 0.08; DF = 6,35
[d] n= 6
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 1.49; p = 0.21; DF = 6,35
[n.s]= not significant.

Table 3-15. Relative Levels of Medaka Whole Brain p450Aromatase mRNA after 21d Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative p450 mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	0.007	0.001	0.0009
Surflan™ 2.5 µl/l	0.0011 [n.s]	0.0012 [n.s]	0.0012 [n.s]
Surflan™ 1.3 µl/l	0.13 [n.s]	0.064 [n.s]	0.2 [n.s]
Surflan™ 0.67 µl/l	6.1 [n.s]	10.9 [n.s]	1.3 [n.s]
Oryzalin 1 mg/l	8.8 **	17.2 **	0.4 [n.s]
Oryzalin 0.5 mg/l	0.0012 [n.s]	0.0021 [n.s]	0.00039 [n.s]
Oryzalin 0.25 mg/l	0.00013 [n.s]	0.00024 [n.s]	0.000012 [n.s]

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 2.55; p = 0.03; DF = 6,76
[b] n=12
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 2.82; p = 0.02; DF = 6,35
[d] n= 6
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 1.28; p = 0.30; DF = 6,35
[n.s]= not significant.
* $p < 0.05$. Not significant by the criterion for statistical significance ($p < 0.008$) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.
** $p < 0.005$

Table 3-16. Relative Levels of Medaka Gonad p450Aromatase mRNA after 21d Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative p450 mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	1.8	1.5	2.0
Surflan™ 2.5 µl/l	3.7 [n.s]	7.4 [n.s]	0.08 [n.s]
Surflan™ 1.3 µl/l	0.9 [n.s]	1.7 [n.s]	0.005 *
Surflan™ 0.67 µl/l	1.0 [n.s]	2.1 [n.s]	0.03 *
Oryzalin 1 mg/l	0.4 [n.s]	0.7 [n.s]	0.002 [n.s]
Oryzalin 0.5 mg/l	0.4 [n.s]	0.7 [n.s]	0.2 [n.s]
Oryzalin 0.25 mg/l	3.4 [n.s]	6.2 [n.s]	0.005 [n.s]

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 1.00; p = 0.43; DF = 6,75
[b] n=12
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 1.21; p = 0.33; DF = 6,35
[d] n= 6
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 1.98; p = 0.10; DF = 6,35
[n.s]= not significant.
* $p < 0.05$. Not significant by the criterion for statistical significance ($p < 0.008$) established by use of the Bonferroni correction for multiple comparisons; data are included to indicate that a value approached statistical significance.

Table 3-17. Relative Levels of Medaka Liver p450Aromatase mRNA after 21d Exposure to Surflan™ or Oryzalin. The tests of significance are Kruskall-Wallace one-way ANOVA nonparametric tests of differences in the medians between the individual treatment and control groups. All α values for determining statistical significance were adjusted by a Bonferroni correction to account for multiple comparisons.

Exposure Concentration	Mean Relative p450 mRNA Levels		
	Females and Males [a,b]	Females[c,d]	Males[d,e]
Control	3.7	3.4	3.9
Surflan™ 2.5 µl/l	3.9 [n.s.]	7.8 [n.s]	0.0 [n.s]
Surflan™ 1.3 µl/l	9.0 [n.s]	9.0 [n.s]	10.8 [n.s]
Surflan™ 0.67 µl/l	0.0 [n.s]	0.0 [n.s]	0.0 [n.s]
Oryzalin 1 mg/l	24.5 [n.s]	3.5 [n.s]	45.6 [n.s]
Oryzalin 0.5 mg/l	168.8 [n.s]	337.4 [n.s]	0.2 [n.s]
Oryzalin 0.25 mg/l	20.6 [n.s]	18.3 [n.s]	3.9 [n.s]

[a] Kruskall-Wallace ANOVA results for Females and Males: F ratio, 1.06; p = 0.40; DF = 6,69
[b] n=12
[c] Kruskall-Wallace ANOVA results for Females: F ratio, 1.24; p = 0.32; DF = 6,33
[d] n= 6
[e] Kruskall-Wallace ANOVA results for Males: F ratio, 1.86; p = 0.12; DF = 6,29
[n.s]= not significant.

SUMMARY

The three criteria established in the introduction as indicative of an endocrine disrupting compound are binding with the estrogen receptor, activation of an estrogen response element on the DNA, and production of estrogen regulated gene products such as choriogenin and vitellogenin. By all three criteria, both Surflan™ and oryzalin are can be considered to be endocrine disruptors. Although Surflan™ did not meet all criteria by failing to competitively displace estrogen from it's receptor, Surflan™ both interacts with an ERE and induces choriogenins. The inactivity of Surflan™ in the competitive binding assay may be due to any of a number of factors; the most likely explanation is that Surflan™ is an emulsion and assay condition may have limited the amount of Surflan™ in solution available for ER binding.

One of the interesting aspects of the response of the medaka to exposure to both Surflan™ and oryzalin is that while there was often no response at high doses, there was a response at lower doses. This sort of response indicates that there is probably a U-shaped or nonmonotonic dose response relationship – a form of response that has been noted for other endocrine disruptors (see following). At the request of the U.S. Environmental Protection Agency (U.S. EPA), the National Toxicology Program organized an independent and open peer review to evaluate the scientific evidence on low-dose effects and nonmonotonic dose-response relationships for endocrine-disrupting chemicals in mammalian species. For this peer review, "low-dose effects" referred to biologic changes that occur in the range of human exposures or at doses lower than those typically used in the standard testing paradigm of the U.S. EPA for evaluating reproductive and developmental toxicity. The demonstration that an effect is adverse was not required because in many cases the long-term health consequences of altered endocrine function during

development have not been fully characterized. A unique aspect of this peer review was the willing submission of individual animal data by principal investigators of primary research groups active in this field and the independent statistical reanalysis of selected parameters prior to the peer review meeting by a sub-panel of statisticians. The expert peer-review panel (the panel) also considered mechanistic data that might influence the plausibility of low-dose effects and identified study design issues or other biologic factors that might account for differences in reported outcomes among studies. The panel found that low-dose effects, as defined for this review, have been demonstrated in laboratory animals exposed to certain endocrine-active agents. In some cases where low-dose effects were reported, there were not classic dose-response curves although the effects varied with the end point and dosing regimen. Typically, these effects were linear at low-dose appearing to reach a threshold, or generally non-monotonic. The findings of the panel indicate that the current testing paradigm used for assessments of reproductive and developmental toxicity should be revisited to see whether changes are needed regarding selection, animal-model selection, age when animals are evaluated, and the end points being measured following exposure to endocrine-active agents.

In female teleosts such as medaka, estrogen regulates vitellogenesis and choriogenin synthesis (Chester-Jones et al. 1987, Murata et al. 1994, 1997), sexual maturation, and reproductive behavior (Gray et al. 1999, Bjerselius et al. 2001). In all vertebrates, estrogen plays a number of critical roles in sexual differentiation of the brain (Chester-Jones et al. 1987). Although estrogens have typically been considered to be primarily female reproductive hormones, data have recently been developed which indicate estrogen has a critical role in male reproduction as well. In mammals, estrogen regulates testicular androgen production by blocking synthesis of

the 17-alpha hydroxylase-C17-20 lyase complex (Dufau 1988). Equally important, estrogen receptors (ER) have been detected in testes and sperm of humans, rodents, and other vertebrate species (Kato et al. 1974, Cooke et al. 1991, Murphy et al. 1980, Mak et al. 1983a,b, Callard and Mak 1985). In rodents and humans, those ERs are of two forms; ER-alpha and ER-beta. Directed mutation of ER-alpha yielded male mice that were infertile, with sperm of young adults unable to fertilize eggs *in vitro* (Mahato et al. 2001). Although ER-beta does not appear to have a critical role in mammalian male reproduction, the number, identity, and specific function of the ER(s) in medaka remain uncertain, with only a single ER identified to date (Kawahara and Yamashita 1999).

The ER, present in target tissues of pituitary, brain, liver, and reproductive tract, is a transcription factor activated by binding of ligand i.e., estrogens or estrogen-agonists; that complex subsequently interacts with an estrogen response element(s) (ERE) proximal to an estrogen-regulated gene (Klinge 2001). The ligand•ER•ERE interaction functions to regulate the transcription rate of specific genes (Pakdel et al. 1991). Estrogen induces the synthesis of its own receptor, and estrogen-responsive tissues are regulated both by alterations in receptor number and in plasma estrogen concentration (Scott and Sumpter 1989, Campbell et al. 1994).

Estrogen is synthesized locally in the ovaries (Chester-Jones et al. 1987) and brain (Pasmanik and Callard 1988) of teleosts by the conversion of androgens to estrogen. That metabolic conversion is mediated by cytochrome p450 aromatase (aromatase), an enzyme that catalyzes the aromatization of the A-ring of C-19 androgen precursors to C-18 estrogens (Chester-Jones et al. 1987). Conversion of testosterone to estradiol by aromatase is critical to the biological activity

of testosterone in the central nervous system (McEwen et al. 1979), and brain aromatase appears to be localized to those regions of the brain involved in reproduction and sexual behavior (McEwen et al. 1979). In mammals, aromatase is expressed in testicular Sertoli and Leydig cells (Valladares and Payne 1979). Aromatase is also present in spermatogonia and epididymal sperm. Spermatogenesis was disrupted in mice lacking a functional aromatase gene, resulting in the arrest of early spermiogenesis and ultimately impairing fertility (Robertson et al. 1999).

Elevated levels of estrogens have detrimental effects on development and function of the male reproductive tract. Estrogen exposures during the perinatal period resulted in structural abnormalities and somatic cell tumors in the reproductive tract of mice and humans (Toppari et al. 1996, Newbold 1998). In non-mammals, estrogen receptor agonists adversely affect reproductive function (Guillette et al. 1994, MacLatchy and Van Der Kraak 1995).

Impaired Leydig cell development and reduced Sertoli cell numbers have been observed in mice treated with the pharmaceutical estrogen ethinyl estradiol (Yasuda et al. 1985). In teleosts, exogenous estrogens lowered basal testosterone levels (Trudeau et al. 1991), and exogenous estradiol significantly inhibited testicular growth (Billard et al. 1981). In rainbow trout, exposure to environmental estrogens induced testicular atrophy and impaired spermatogenesis, while in medaka, exposure to exogenous estrogen or to estrogenic chemicals reduced fecundity (Jobling et al. 1996, Nimrod and Benson 1998, Gronen et al. 1999, Gray et al. 1999)

Estrogen and estrogenic chemicals are critical regulators of reproduction in male and female vertebrates. The biological actions of estrogen, mediated by the ER, indicate that changes in ER

expression have the potential to alter the transcription of estrogen-sensitive genes. Likewise, aromatase – as the primary source of endogenous estrogens – is clearly a key component in the control of estrogen-regulated gene transcription. Considered singly or in combination, alterations in expression of the ER and/or aromatase have the potential to disrupt normal reproductive function. Our reproductive study, in which we observed oryzalin- and Surflan™-induced increases in the mean daily percentage of non-fertilized eggs, and a decrease in the daily total production of fertilized eggs, are consistent with previously documented effects of estrogen or estrogen agonists on reproduction. Although we cannot yet causally link our observations of the direct effects of oryzalin and Surflan™ on ER and aromatase mRNA to specific mechanisms of reproductive toxicity, changes in expression of the ER and p450aromatase clearly have considerable explanatory potential in understanding the adverse effects on medaka reproduction.

Relative use of oryzalin throughout the state

Oryzalin is a widely used herbicide throughout California and is used on a variety of crops ranging from orchards to vineyards as well as in urban areas for the maintenance of weeds. Use from 1991-2000 ranged from 814,397 lbs on 403,701 acres in 1998 to a low of 456,521 lbs on 219,040 acres in 2000, and the application rate is relatively constant at about 1.6 to 1.7 lbs per acre (Pesticide Action Network, http://www.pesticideinfo.org/Search_Use.html). By comparison, atrazine use over the same period ranged from 36,078 lbs on 31,571 acres in 1995 to 69,549 lbs on 37,371 acres in 1999, with usage ranging from 1.2 lbs per acre to 1.7 lbs per acre. Diuron was used at amounts ranging from 916,017 lbs on 390,505 acres to 1,504,268 lbs on 860,895 acres, with application rates ranging from 0.7 to 1.3 lbs per acre. Glyphosate use ranged

from 2,702,425 lbs on 2,156,805 acres to 4,641,557 lbs on 3,693,481 acres with application rates ranging from 0.7 to 0.9 lbs per acre. According to statistics published on the PAN website, several herbicides have much greater use throughout the state in terms of total pounds used including molinate, glyphosate, diuron, thiobencarb, trifluralin, and more recently propanil. The application rate of oryzalin is high relative to other pesticides although herbicides such as propanil have application rates that range from 4 to almost 6 lbs per acre, far higher than the application rates for oryzalin.

Synergism and additivity with other compounds

The toxicological and ecological consequences of endocrine disrupting chemicals are currently under intense scientific scrutiny (Guilette and Gunderson 2001, Melnick et al. 2002, Hayes et al. 2002) because of the documented ability of these substances to adversely affect the reproductive health of diverse species of animals (Bowerman et al. 1995, Jobling et al. 1996, Gray and Metcalfe 1997, Blake and Boockfor 1997, Bookfor and Blake 1997, Van der Kraak 1998). The human experience with the synthetic estrogen, diethylstilbestrol (McLachlan and Dixon 1976), and extensive data from laboratory animals and wildlife (Billard et al. 1981, Sharpe et al. 1995, Jobling et al. 1996, Gray and Metcalfe 1997, Blake and Boockfor 1997, Boockfor and Blake 1997, Gray et al. 1999a,b, Gronen et al. 1999, Foran et al. 2000, Cheek et al. 2001) indicate that impaired reproduction is a common effect of exposure to these substances. The extent to which population-level impacts on wildlife are occurring, or may occur in the future from exposure to these substances, is uncertain. However, the possibility that at least some of these substance can act additively (Weise et al. 1997) or synergistically (Arnold et al. 1996) suggests that endocrine-

disrupting chemicals present as environmental contaminants may pose risks to the long term viability of multiple species.

Conservation of effects across species

In all vertebrate species studied to date, estrogen is synthesized by p450aromatase conversion of testosterone, and it is clear that ER proteins mediate effects of endogenous estrogens on target tissues. Many exogenous estrogenic chemicals, including oryzalin and Surflan™, appear to act as estrogen agonists, and thus have the capacity to act biologically as estrogens and to modulate expression of estrogen-dependent genes. Although it may be most appropriate to extrapolate the specific effects of oryzalin and Surflan™ on medaka only to other teleosts, the estrogen receptor is closely conserved among all vertebrates (Le Drean et al, 1995). Likewise, there appears to be considerable structural organization and regulatory homology of the p450arom gene across species (Tanaka et al. 1995). Consequently, direct or indirect interaction or perturbation of estrogen receptor or p450arom levels in one vertebrate species clearly indicates the likelihood of similar action in others. The similarity of function of endogenous sex steroids and the common functions of estrogen receptor and p450 aromatase among different groups of vertebrates suggests that chemicals such as oryzalin and Surflan™ pose a potential hazard not only to fish such as medaka, but to wildlife species and to humans as well.

Environmentally relevant concentrations

One of the main unresolved questions raised by our results is if the effects found in this investigation are occurring at concentrations that are environmentally relevant, i.e., are concentrations found in natural systems comparable to the dose levels used in this study. Because herbicides are assumed to be essentially nontoxic to mammals and birds, and only slightly toxic to fish, few studies have attempted to measure the concentrations of Surflan™ or oryzalin in the field. The only study with reliable data is the study currently being conducted by T. Young on the runoff of oryzalin from sites near Tolay Creek and the Eel River. Over two years, concentrations of oryzalin in storm water runoff from the Tolay Creek site averaged 2.2 μg/L and 0.7 μg/L (EMC) while concentrations at the Eel River site averaged 12.6 μg/L and 17.9 μg/L (EMC). Single storm EMC concentrations were found to be as high as 43 μg/L. Effects were seen in this study at concentrations as low as 250 μg/L, well above the levels measured by Young et al. in their field study. Because our studies were conducted after a general dose-range finding process, and because it was unknown at the time the studies were initiated what the environmentally relevant concentrations would be, no attempt was made to test for effects at concentrations as low as those seen in the field. Consequently, it is important that our studies be repeated at the low concentrations detected by Young et al. in their study.

It is also emphasized that herbicides in general are persistent, soluble, and resistant to degradation. Oryzalin is relatively less soluble than compounds such as the triazine herbicides (e.g., atrazine, simizine), but appears to be as resistant to degradation indicating that small amounts of this chemical may persist in the environment for periods of time ranging from days to months. ED effects of oryzalin were seen in this investigation at exposure times as short as 24 hours. At the least, oryzalin could remain in the environment for the length of time the

exposures in this experiment were performed (maximum of 21 days). Even if the concentrations seen by Young et al. (unpubl.) are common in the field, the persistence of the compound low concentrations of Surflan™ (oryzalin) over longer periods of time may also give rise to effects in fish. Low dose-long term exposure experiments should be performed to determine if the ED effects could be observed under environmentally relevant exposure scenarios.

Future investigations

Characterizing the specific site(s) of action, and working towards a comprehensive understanding of the mechanistic basis of toxicity of endocrine-disrupting chemicals are necessary if we are to understand and address the true risks posed by these chemicals. Alterations of the endocrine system may be multifaceted and biologically complex. Our data clearly demonstrate that oryzalin and Surflan™ are estrogenic reproductive toxins, whose overt effects appear to be due to induction of gonadal lesions in both genders, as well as to disruption of spermatogenesis in males. However, it is important to note that the other observations documented in that portion of our work, i.e., an increase in the frequency of occurrence of non-fertilized eggs, and a decrease in the total number of fertilized eggs produced, may have been partially or entirely caused by behavioral consequences of exposure.

Because our data represent the first report of the endocrine-disrupting actions of oryzalin and Surflan™, we have not had an opportunity to determine whether there are effects on behavior or effects on developing embryos, nor do we have information on the biological consequences of exposure to oryzalin and Surflan™ at lower concentrations and longer exposure times more

typical of environmental exposures than we used in these studies. Similarly, our results on the ability of oryzalin and Surflan™ to modulate expression of ER and p450arom mRNA, and to do so on a tissue- and time-specific basis, pose a host of compelling questions. For example, do the effects on p450arom of oryzalin and Surflan™ affect tissue-specific or circulating levels of estradiol? Are there feedback effects of p450arom that may alter testosterone synthesis, either locally in gonads or in the brain? We do not know the biological significance of p450arom in medaka liver, nor do we understand whether its induction leads to enhanced degradation of testosterone and/or increased production of estradiol and if so, whether these effects are restricted to the liver or if they extend to circulating levels of these hormones.

Given that oryzalin is a dinitrobenzensulfonamide – a class of chemical not previously identified as estrogenic, it is difficult to hypothesize as to what the specific molecular and biochemical actions of oryzalin will prove to be. Indeed, one of the intriguing aspects of oryzalin is that conventional toxicity data led the US EPA to classify it as practically non-toxic to mammals (e.g., Kidd and James 1991). Our data raise questions concerning the safety of oryzalin and Surflan™ to mammals, while clearly indicating their adverse effects on fish. The effects identified here have not been previously addressed experimentally for oryzalin and Surflan™, and point to the need to expand the scope of testing prior to marketing of herbicides.

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Appendix I. Physical Properties of Oryzalin

Appearance: Technical oryzalin is a bright yellow-orange crystalline powder [1].
CAS Number: 19044-88-3
Molecular Weight: 346.36
Water Solubility: 2.5 mg/L @ pH 7 and 25 C [1]
Solubility in Other Solvents: v.s. in organic solvents such as acetone, methanol, and acetonitrile; s.s. in benzene and xylene; i.s. in hexane [1]
Melting Point: 141-142 C [1]
Vapor Pressure: <0.013 mPa @ 30 C [1]
Partition Coefficient: 3.7340 @ pH 7 [1]
Adsorption Coefficient: 600 [11]